RAQUEL DE ARRUDA LEME

# EPIDEMIOLOGIA MOLECULAR DE *TORQUE TENO SUS VIRUS* EM REBANHOS SUINÍCOLAS BRASILEIROS

Londrina
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Dissertação apresentada ao Programa de Pós-graduação em Ciência Animal – área de concentração Sanidade Animal – da Universidade Estadual de Londrina como requisito parcial para a obtenção do título de Mestre em Ciência Animal.

**Orientador**: Prof. Dr. Amauri Alcindo Alfieri

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Ao meu esposo, Ricardo,
e filhos, Luiza e João Pedro.
Aos meus pais,
Luiz Carlos e Mari Angela,
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**“Viva como se fosse morrer amanhã.**
**Aprenda como se fosse viver para sempre”**

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LEME, Raquel de Arruda. **Epidemiologia molecular de *Torque teno sus virus* em rebanhos suinícolas brasileiros**. 2013. 78f. Dissertação (Mestrado em Ciência Animal; Área de Concentração: Sanidade Animal) – Universidade Estadual de Londrina, Londrina. 2013.

## RESUMO

O *Torque teno sus virus* (TTSuV) pertence à família *Anelloviridae*, gêneros *Iotatorquevirus* para as espécies *torque teno sus virus 1a* (TTSuV1a) e *torque teno sus virus 1b* (TTSuV1b), e *Kappatorquevirus*, para a espécie *torque teno sus virus k2* (TTSuVk2). Este estudo visou avaliar a infecção natural pelo TTSuV nos rebanhos suinícolas brasileiros. Para isso foram realizados três estudos, utilizando a técnica da reação em cadeia da polimerase (PCR) que teve como alvo a região não traduzida (UTR) do genoma viral. O primeiro estudo teve como objetivo avaliar a frequência da infecção natural pelo TTSuV nas três principais regiões produtoras de suínos do Brasil. Amostras de fezes (*n*=135) de leitões de maternidade foram analisadas para a presença do vírus. Foi possível demonstrar que a infecção tanto pelo TTSuV1 quanto pelo TTSuVk2 ocorre em leitões de 1 a 3 semanas de idade nas regiões Sul, Sudeste e Centro-oeste do Brasil, com frequências de ocorrência que variaram de 40,5% a 51,1% para o TTSuV1 e de 8,8% a 24,3% para o TTSuVk2. O segundo estudo teve como objetivo avaliar a distribuição da infecção pelo TTSuV1 e TTSuVk2 nas diferentes categorias do ciclo de produção de suínos em rebanhos do estado do Paraná. Foram analisadas amostras de fezes de animais em maternidade (1 a 3 semanas, *n*=35), creche (4 a 8 semanas, *n*=43), terminação (9 a 24 semanas, *n*=71) e de reprodutores (*n*=41). Os resultados revelaram que a infecção pelo TTSuV está disseminada em todo o ciclo de produção de suínos nas granjas paranaenses. As frequências de detecção do vírus entre as categorias variaram de 5,6% a 28,6% (TTSuV1), 8,6% a 54,9% (TTSuVk2) e 7,3% a 29,5% (TTSuV1+TTSuVk2 em co-infecção). Pode-se observar que animais jovens são mais comumente infectados pelo TTSuV1, enquanto suínos na fase de terminação são mais comumente infectados pelo TTSuVk2. O objetivo do terceiro estudo foi avaliar a presença do TTSuV em amostras de orgãos e, concomitantemente, a viremia em suínos adultos assintomáticos de abatedouro e avaliar a presença de infecção simultânea por cepas distintas dos mesmos gêneros de TTSuV. Amostras pareadas (*n*=116) de fragmentos de orgãos (fígado ou pulmão) e soro foram analisadas para a presença do vírus. As cepas de TTSuV encontradas em amostras de orgãos apresentaram diferenças na similaridade dos fragmentos de nucleotídeos amplificados quando comparadas aos fragmentos de nucleotídeos de cepas obtidas a partir das amostras de soro nos mesmos animais e entre os animais, o que demonstra que infecções mistas simultâneas por cepas distintas de TTSuV1 e TTSuVk2 são comuns em suínos em idade de abate de rebanhos paranaenses. Portanto, foi possível demonstrar que a infecção pelo TTSuV está disseminada nos rebanhos suinícolas do Brasil e que leitões e suínos adultos são mais frequentemente infectados pelo TTSuV1 e TTSuVk2, respectivamente. Foi demonstrado que as estirpes de TTSuV1 e TTSuVk2 circulantes no Brasil apresentam importante variabilidade genética e que infecções simultâneas por cepas distintas dos mesmos gêneros de TTSuV são comuns.

**Palavras-chave**: *Anelloviridae. Iotatorquevirus. Kappatorquevirus.* Suínos. Fezes.

LEME, Raquel de Arruda. **Molecular epidemiology of *Torque teno sus virus* in Brazilian pig herds**. 2013. 78f. Dissertation (Master Degree in Animal Science) – Universidade Estadual de Londrina, Londrina. 2013.

## ABSTRACT

*Torque teno sus virus* (TTSuV) belongs to the family *Anelloviridae*, genera *Iotatorquevirus* for the species *torque teno sus virus 1a* (TTSuV1a) and *torque teno sus virus 1b* (TTSuV1a), and *Kappatorquevirus* for the species *torque teno sus virus* k2 (TTSuVk2). This study purposed to evaluate the natural infection by TTSuV in Brazilian pig herds. It was realized three studies by using the polymerase chain reaction (PCR) assay targeting the untranslated region (UTR) of the viral genome. The first study aimed to evaluate the natural infection by TTSuV in the three major pig-producing regions of Brazil. Piglet fecal samples (*n*=135) were analyzed for the presence of the virus. It was possible to demonstrate that TTSuV1 and TTSuVk2 infect piglets of 1 to 3 weeks of age in the South, Southeast, and Midwest regions of Brazil, with frequencies varying from 40.5% to 51.1% for TTSuV1 and 8.8% to 24.3% for TTSuVk2. The second study aimed to evaluate the distribution of TTSuV1 and TTSuVk2 in the different stages of pig production cycle in herds of Paraná state, Brazil. It was analyzed fecal samples of piglets (1 to 3 weeks old, *n*=35), weaned pigs (4 to 8 weeks old, *n*=43), finisher pigs (9 to 24 weeks old, *n*=71), and breeders (*n*=41). The results revealed that TTSuV infection has disseminated in all stages of pig production cycle in farms of Paraná state. The frequencies of virus detection between categories varied from 5.6% to 28.6% (TTSuV1), 8.6% to 54.9% (TTSuVk2), and 7.3% to 29.5% (TTSuV1+TTSuVk2 in co-infection). Piglets were more frequently infected with TTSuV1, while finisher pigs were more frequently infected with TTSuVk2. The goal of the third study was to evaluate the presence of TTSuV in organs and concomitant viremia in healthy pigs at slaughter age and to evaluate the presence of simultaneous infection by distinct strains of the same TTSuV genus. Paired samples (*n*=116) of organ (liver or lung) fragments and serum were analyzed for the virus presence. The strains of TTSuV detected in the organ samples presented differences in the amplified nucleotide sequence similarities when compared to amplified nucleotide sequences of strains obtained from serum samples between and within the pigs, which demonstrates that simultaneous mixed infections by distinct TTSuV1 and TTSuVk2 strains are common in pig herds of Paraná state. In conclusion, it was possible to demonstrate that TTSuV infection has disseminated in Brazilian pig herds and that piglets and finisher pigs are more frequently infected with TTSuV1 and TTSuVk2, respectively. It was demonstrated that TTSuV1 and TTSuVk2 strains circulating in Brazil state presents important genetic variability and that simultaneous infection by distinct strains of TTSuV are common.

**Key Words**: *Anelloviridae. Iotatorquevirus. Kappatorquevirus.* Swine. Feces.

# LISTA DE ILUSTRAÇÕES

# LISTA DE TABELAS

# LISTA DE ABREVIATURAS

| | |
|---|---|
| aa | Amino acid (Aminoácido) |
| ABIPECS | Brazilian Association of Producer and Exporter Industry of Pig Meat (Associação Brasileira da Indústria Produtora e Exportadora de Carne Suína) |
| CAV | Chicken anemia virus (Vírus da anemia infecciosa das galinhas) |
| CSFV | Classical swine fever virus (Vírus da peste suína clássica) |
| HEV | Hepatitis E virus (Vírus da hepatite E) |
| ICTV | International Committee on Virus Taxonomy (Comitê Internacional de Taxonomia Viral) |
| nPCR | Nested-polymerase chain reaction (Segunda reação em cadeia da polimerase) |
| nt | Nucleotide (Nucleotídeo) |
| ORF | Open reading frame (Fase aberta de leitura) |
| PCR | Polymerase chain reaction (Reação em cadeia da polimerase) |
| PCV1 | Porcine circovirus 1 (Circovírus suíno tipo1) |
| PCV2 | Porcine circovirus 2 (Circovírus suíno tipo 2) |
| PCVAD | Porcine circovirus associated disease (Doença associada ao circovírus suíno) |
| PDNS | Porcine dermatitis and nephropaty syndrome (Síndrome da dermatite e nefropatia dos suínos) |
| PMWS | Porcine post-weaning multisystemic wasting syndrom (Síndrome multissistêmica de definhamento dos suínos) |
| PRDC | Porcine respiratory disease complex (Complexo doença respiratória dos suínos) |
| PRRSV | Porcine reproductive and respiratory syndrome virus (Vírus da síndrome reprodutiva e respiratória dos suínos) |
| RCA | Rolling circle amplification (Amplificação por círculo rolante) |
| RCR | Rolling circle replication (Replicação por círculo rolante) |
| SPF | Specific pathogen free (Livre de patógenos específicos) |
| ssDNA | Single-stranded DNA (DNA de fita simples) |
| TTV | Torque teno virus (Torque teno vírus) |
| TTSuV | Torque teno sus virus (Torque teno sus vírus) |
| UTR | Untranslated region (Região não traduzida) |

# SUMÁRIO

# 1 REVISÃO DE LITERATURA

## 1.1 – INTRODUCTION

Torque teno virus (TTV) was first isolated in a Japanese patient as the causative agent of an acute post-transfusion hepatitis of unknown etiology (NISHIZAWA et al., 1997). After the TTV discovery, the virus infection has been demonstrated in different human population around the world (ABE et al., 1999; LEARY et al., 1999).

Because of the genome structure of TTV formed by a single-stranded DNA (ssDNA) of negative polarity to be very similar to the chicken anemia virus (CAV), family *Circoviridae*, genus *Gyrovirus*, the TTV has been initially included as the new member of this family, and the first human circovirus identified (HINO; MIYATA, 2007). Further studies have shown significant differences in the nucleotide (nt) sequence similarities between TTV and other circovirus, and TTV has been proposed to belong to a new virus family, tentatively named *Circinoviridae* (from the Latin *circinatio*, meaning "the describing of a circle") (MUSHAHWAR et al., 1999), *Paracircoviridae* (TAKAHASHI et al., 2000) and TTV family (TANAKA et al., 2001).

Torque teno virus name derives from the Latin, *torques tenuis*, meaning "thin necklace", as a reference to the circular structure of the viral genome (HINO; MIYATA, 2007). Nowadays, the International Committee on Taxonomy of Viruses (ICTV) has officially classified the TTV as belonging to the family *Anelloviridae* (from the Latin, "ring"). Human TTV is classified into the genus *Alphatorquevirus*, which clusters 29 genetically distinct species (ICTV, 2012). Despite the genetic variability, the viral genome organization is well-conserved (BIAGINI; DE MICCO, 2008).

The TTV is spherical, non-enveloped, with a diameter of 30-32 nm, genomic length of 3.8 kb, and density of 1.31-1.35 g/cm³ (ITOH et al., 2000). The ssDNA genome of the TTV is divided into two regions: a coding sequence of approximately 2.6 kb, that is extremely variable; and a conserved untranslated region (UTR) of approximately 1.2 kb (BIAGINI; DE MICCO, 2008).

The coding sequence has two major open reading frames (ORFs). The ORF1, with approximately 770 amino acids (aa), is assumed to encode a structural and replication-associated protein (BIAGINI; DE MICCO, 2008). Differences found in the glycosylation pattern of the ORF1 in distinct isolates were suggested to result in considerable

differences in the antigenicity and other biological properties of the ORF1-encoded protein (HIJIKATA; TAKAHASHI; MISHIRO, 1999).

The ORF2 has approximately 202 aa that encode a non-structural protein with putative phosphatase activity, probably involved in the viral replication (BIAGINI; DE MICCO, 2008). The protein enconded by ORF2 has been suggested to inhibit cell production of the pro-inflammatory mediators involved in multiple signal transduction pathways that active inflammations, regulating the innate and adaptive immune responses in infected hosts (ZHENG et al., 2007).

Two additional reading frames (ORF3 and ORF4) were suggested to exist, since at least three species of spliced mRNA molecules were identified (BIAGINI; DE MICCO, 2008).

The UTR of TTV genome presents a GC rich stretch, a TATA-box element, and several regulatory elements that are believed to participate as promoter and enhancer of viral replication (SUZUKI et al., 2004).

Human TTV is able to replicate in some culture cells, but this tool has been used only to determine the full-genome sequences of TTVs (LEPPIK et al., 2007). A cell culture system that effectively supports TTV replication and release infectious TTV particles is not available (DAVIDSON; SHULMAN, 2008) and this fact prevents the conduction of depth studies on the biological properties of the virus, such as the replication mechanism and the cell host-virus interaction (BIAGINI; DE MICCO, 2008).

Studies have detected *in vivo* replicative viral DNA forms in the bone marrow of humans (OKAMOTO et al., 2000b) and evidenced TTV replication in activated peripheral blood mononuclear cells (MAGGI et al., 2001; ZHONG et al., 2002). TTV DNA in replicative forms and TTV mRNA have also been detected in different tissues, like lymph node, muscle, thyroid gland, lungs, liver, spleen, pancreas, and kidneys (OKAMOTO et al., 2001a).

The rolling circle replication (RCR) mechanism has been thought to be the way the virus replicates, since conserved Rep protein motifs have been identified in the ORF1 translation products of TTV isolates (OKAMOTO et al., 2000b). However, the possibility of TTV to replicate by machinery with poor or no proofreading activity has been considered, due to the high genetic diversity of the virus (BENDINELLI et al., 2001).

The TTV infection has been demonstrated in different human population around the world (ABE et al., 1999; LEARY et al., 1999), including those at risk of parenteral

transmission (drug users, HIV-positive patients, and hemophiliacs), but also in individuals with no apparent pathology, such as blood donors (BIAGINI; DE MICCO, 2008).

The prevalence between patients with liver, pulmonary or autoimmune diseases, hematological disorders, cancer, or HIV-positive, for example, is higher than in healthy individuals (GERGELY JR; PERL; POÓR, 2006; BIAGINI; DE MICCO, 2008; MAGGI et al., 2010). However, the fact that the active infection is highly prevalent also in apparent healthy individuals reinforces the hypothesis that the virus might be devoid of pathogenic potential (WATANABE et al., 2005).

Despite the increasing amount of data, many aspects of the biology of TTV, like replication methods, antibodies induction against TTV-produced proteins, significance of its global presence and high genomic variability, cell tropism, regulation of replication and transcription have not yet been elucidated (GERGELY JR; PERL; POÓR, 2006).

## 1.2 – TTV IN ANIMALS

After the human TTV reports, studies have evaluated the TTV infection in many other animal species. The presence of TTV has been demonstrated in non-human primates, tupaias (small mammals from forests of Asian countries), farm animals (chicken, pig, cow, and sheep), domestic animals (cat and dog), camels, and sea lion (LEARY et al., 1999; OKAMOTO et al., 2000a; OKAMOTO et al., 2001b; OKAMOTO et al., 2002; AL-MOSLIH; PERKINS; HU, 2007; NG et al., 2009).

The TTV infection has been demonstrated to be species-specific (OKAMOTO et al., 2002), although it has also been demonstrated that cross-species infection may eventually occur (OKAMOTO et al., 2000a). The classification of the genus depends on the viral host species (Table 1).

**Table 1** – Official classification for the family *Anelloviridae.*

| Genus | Species (nº of) | Host |
|---|---|---|
| *Alphatorquevirus* | *Torque teno virus* (29) | Human, chimpanzee, African monkey |
| *Betatorquevirus* | *Torque teno mini virus* (12) | Human, chimpanzee, African monkey |
| *Deltatorquevirus* | *Torque teno tupaia virus* (1) | Tupaia (*Tupaia belangeri*) |
| *Epsilontorquevirus* | *Torque teno tamarin virus* (1) | Tamarin (*Saguinus oedipus*) |
| *Etatorquevirus* | *Torque teno felis virus* (2) | Cats |
| *Gammatorquevirus* | *Torque teno midi virus* (15) | Human, chimpanzee, African monkey |
| *Iotatorquevirus* | *Torque teno sus virus 1* (2) | Pigs |
| *Kappatorquevirus* | *Torque teno sus virus k2* (1) | Pigs |
| *Lambdatorquevirus* | *Torque teno zalophus virus* (1) | Sea lions |
| *Thatatorquevirus* | *Torque teno canis virus* (1) | Dogs |
| *Zetatorquevirus* | *Torque teno douroucouli virus* (1) | Douroucouli (*Aotes trivirgatus*) |

**Source**: ICTV (2012).

## 1.3 – TTV IN PIGS

### 1.3.1 – Classification

Pigs were first described as positive for TTV by Leary et al. (1999) in the United States. Okamoto et al. (2002) in Japan first characterized the entire genome sequence of the virus and proposed a prototype (Sd-TTV31) as a model for researches. Niel, Diniz-Mendes and Devalle (2005), in Brazil, isolated two clones of porcine TTV (Sd-TTV1p and Sd-TTV2p). The Sd-TTV1p showed 69.6% of nt similarity with the existing prototype (Sd-TTV31). The Sd-TTV2p showed very lower nt similarity when compared to Sd-TTV1p and Sd-TTV31 (43.4% and 45.1%, respectively). Then, it was suggested that Sd-TTV31 were the prototype for the genogroup 1, and Sd-TTV2p a new prototype for the genogroup 2.

In 2011, a new classification was established that designated the genogroups as distinct species in two different genus: *Torque teno sus virus 1a* (TTSuV1a) and *Torque teno sus virus 1b* (TTSuV1b), which are represented by Sd-TTV31 and Sd-TTV1p, respectively, in the genus *Iotatorquevirus*, and *Torque teno sus virus k2* (TTSuVk2), which is represented by Sd-TTV2p in the genus *Kappatorquevirus* (ICTV, 2012).

In the last year, it has been reported a possible novel TTSuV species, suggestively named TTSuVk2b, in the genus *Kappatorquevirus* (CORNELISSEN-KEIJSERS et al., 2012). However, this is a recent finding and there was no time for an official classification.

### 1.3.2 – Genetic and Morphological Features of TTSuV

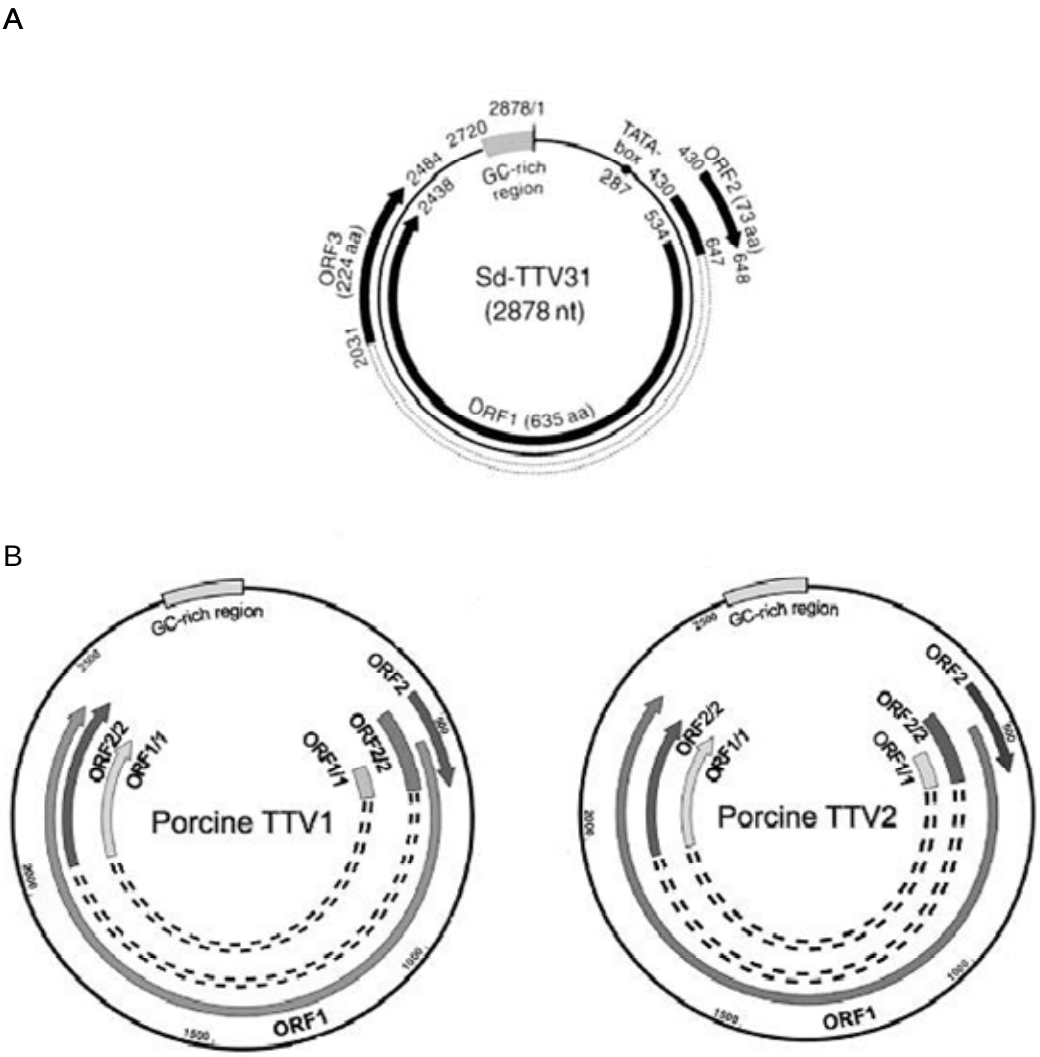
The TTSuV genome has approximately 2.9 kb (2,878 nt) and, like in human TTV, is organized in ORFs and an UTR sequence, considered the most conserved region of the entire genome among all TTVs. The UTR of TTSuV has 823 nt, occupying 29% of the viral genome. The UTR also has a TATA-box and a region rich in GC nt, forming repeated stem-loop structures (OKAMOTO et al., 2002).

As a highly conserved region of the viral genome, the UTR is considered a good marker for molecular diagnosis of TTSuV, because its molecular diversity is sufficient not only for detection, but also for the classification of the virus. The limitation of its use in molecular studies is because the UTR do not permit an assessment of phylogenetic evolution. For this, the use of other regions of the TTSuV genome where there is a great selection pressure would be more useful (SEGALÉS et al., 2009).

The remainder of the TTSuV genome is organized in ORFs. At first, three ORFs were considered – ORF1, ORF2, and ORF3 (OKAMOTO et al., 2002; NIEL; DINIZ-MENDES; DEVALLE, 2005).

Huang et al. (2010a) suggested that the nomenclature used in the ORF classification of human TTV was also followed in the classification of the TTSuV ORFs, with ORF1 and ORF2. According to the authors, after transcription the mRNA suffers splicing and generates new mRNA, named ORF1/1 and ORF2/2. The ORF3 is considered the ORF2/2, since it starts at the same portion of the ORF2 and remains in this same ORF after splicing (Figure 1).

**Figure 1** – Schematic diagram comparing the structure of the TTSuV genome; A) Genomic organization proposed by Okamoto et al. (2002) with the GC nt rich region and the TATA-box shown in the UTR region of the genome; the three ORFs are represented; B) the GC nt rich region is shown, as well as the location of the ORFs according to the new classification proposed by Huang et al. (2010b).




**Source**: Okamoto et al. (2002); Huang et al., (2010b).

The ORF1 probably encodes a protein with structural and replication functions. RCR-associated motifs have been identified in the ORF1 of both TTSuV species; however the RCR motif found in the TTSuV1 ORF1 was not the same of the RCR motif of the TTSuV2 ORF1 (HUANG et al., 2010a; MARTÍNEZ-GUINÓ et al., 2011). Besides, all RCR motifs identified were conserved in the TTSuV1 or TTSuV2 genomes analyzed

(MARTÍNEZ-GUINÓ et al., 2011). A conserved arginine N-terminal region has also been identified in the TTSuV ORF1 (HUANG et al., 2010a; MARTÍNEZ-GUINÓ et al., 2011), and are thought to have the DNA binding activity (HUANG et al., 2010a). Segalés et al. (2009) suggested that the ORF1 could be used to evaluate the phylogenetic evolution, since it encodes a capsid protein and is subject to selection pressure, and also to be possibly involved in the mechanism of host immune response escape, having immunogenic characteristics that help the TTSuV to establish persisting infection (HUANG et al., 2010a).

It is believed that the ORF2 encodes a non-structural protein responsible for regulating host gene transcription, translation signal, and cytokine response during the viral replication (HUANG et al., 2010a; CORTEY et al., 2011). TTSuV ORF2 contains a protein-tyrosine phosphatase motif in the N-terminal region that has been presented as a conserved motif, while the rest of the ORF2 aa sequence has not been reported conserved between the virus species (HUANG et al., 2010a; MARTÍNEZ-GUINÓ et al., 2011). It has been suggested that the protein generated from the ORF2 also decreases the expression of inflammatory factors (IL-6, IL-8, COX2) and is involved in the regulation of the innate and adaptive immune responses of the host species. This mechanism would contribute to a possible pathogenesis of TTSuV and consequent disease (ZHENG et al., 2007).

The expression profile and subcellular localization of TTSuV1 and 2 proteins have been evaluated. However, the lack of techniques other than PCR or quantitative PCR and without having an efficient culture system supporting TTSuV replication, which impedes the purification of the virus, has limited TTSuV research to epidemiological studies and, therefore the elucidation of the definitive functional role of TTSuV proteins is still lacking (MARTÍNEZ-GUINÓ et al., 2011).

### 1.3.3 – Epidemiology

The TTSuV infection is widespread in pig herds worldwide, including Japan, China, Thailand, Serbia, Germany, Spain, Canada, the United States, Cuba, and Brazil (NIEL; DINIZ-MENDES; DEVALLE, 2005; GALLEI et al., 2010; SAVIC et al., 2010; PÉREZ et al., 2011; MENG, 2012). The viral DNA has been detected in pig serum, peripheral blood mononuclear cells, semen and fecal samples (OKAMOTO et al., 2002; MCKEOWN et al., 2004; KEKARAINEN; LOPEZ-SORIA; SEGALÉS, 2007; BRASSARD et al., 2008; TSHERING; TAKAGI; DEGUCHI, 2012), and also in tissues, such as liver, spleen, heart,

lungs, kidneys, tonsil, ileum, lymph nodes, brain, and bone marrow (BIGARRÉ et al., 2005; ARAMOUNI et al., 2010; SAVIC et al., 2010).

The prevalence of TTSuV infection in serum samples of pigs from different geographic regions of the world varied between 24% and 100% of animals determined to be positive for the virus (KEKARAINEN; SEGALÉS, 2012). The TTSuV1 and 2 prevalence in serum, peripheral blood mononuclear cells, nasal secretions, stool, and organs samples have been demonstrated to increase with the age of the animals (SIBILA et al., 2009b; ARAMOUNI et al., 2010; JAROSOVA; POGRANICHNIY; CELER, 2011; TSHERING; TAKAGI; DEGUCHI, 2012; XIAO et al., 2012).

The route of TTSuV transmission has been demonstrated to vary. The main transmission route is thought to be the fecal-oral, but other vias also occur and are epidemiologically important. The detection of the virus in pig fecal samples not only suggested that the TTSuV may be transmitted by a fecal-oral route, but also indicated that some farming practices, such as the application of pig manure and irrigation with river water, can be responsible for the introduction and permanence of TTSuV in the environment (BRASSARD et al., 2008).

Kekarainen et al. (2007) demonstrated the presence of TTSuV in boar semen and suggested the venereal via as an important route of infection.

The transmission of TTSuV by in utero route has been shown (POZZUTO et al., 2009), and the TTSuV infection in sow and piglets was demonstrated, reinforcing the possibility that vertical transmission may occur (SIBILA et al., 2009a). This same study showed that piglet-to-piglet transmission is also epidemiologically important. Sibila et al. (2009b) demonstrated the presence of the virus in nasal swabs, which suggests that oro-nasal is another route of transmission.

McKeown et al. (2004), in the United States, suggested that a common source of TTSuV transmission might exist, since there is a globally distribution of the virus without geographical grouping of the isolates. According to the authors, it could be explained by the production of vaccines containing contaminated components of porcine origin or even iatrogenic route.

The existence of contamination of commercial products intended for humans and pigs, as well as enzymes used in laboratories, has been demonstrated in Spain, which reinforces the previously presented hypothesis (KEKARAINEN; MARTÍNEZ-GUINÓ; SEGALÉS, 2009).

Another study revealed the contamination of cell lineages from thirteen different species and three cell lineages of pig origin with TTSuV genomes, as well as one of the tested batches of trypsin, which tested positive for TTSuV1 distinct strains and TTSuV2 at the same time (TEIXEIRA et al., 2011). It was suggested that cell lineages could be a source for contamination of biological products. The authors also evidenced that the virus may be circulating for more than 25 years, once they could detect the TTSuV genome in a frozen cell lineage that had been ampouled in 1985.

Finally, the ways of TTSuV spreading are diverse and the occurrence of both vertical and horizontal transmission are one of the justifications for the high frequency of diagnosis of the virus worldwide and demonstrate that TTSuV is highly infectious (GALLEI et al., 2010).

### 1.3.4 – Pathogenesis

TTSuV has not been definitively associated with a specific pathology and the infection is common to both healthy and diseased pigs (MENG, 2012). It has been demonstrated that the TTSuV might contribute to the expression of other important viral diseases of economic and public health impacts, such as porcine circovirosis, the reproductive and respiratory syndrome, classical swine fever, and hepatitis E in pigs. Animals infected with these viruses and asymptomatic, sub-clinical, or patient (mild symptoms), since co-infected with TTSuV, may initiate or exacerbate the clinical signs (KEKARAINEN; SIBILA; SEGALÉS, 2006; ELLIS; ALLAN; KRAKOWKA, 2008; KRAKOWKA et al., 2008; SAVIC et al., 2010).

Studies of different countries have investigated the prevalence of TTSuV in pigs with and without post-weaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS), both caused by porcine circovirus 2 (PCV2). The results revealed that both TTSuV species were highly prevalent in porcine circovirus associated disease (PCVAD)-affected pigs (KEKARAINEN; SIBILA; SEGALÉS, 2006; TAIRA et al., 2009; ARAMOUNI et al., 2011; PÉREZ et al., 2011; NOVOSEL et al., 2012). Kekarainen, Sibila and Segalés (2006) showed that animals with PMWS had 1.25 times more risk of being infected with TTSuV than pigs without PCV2.

The experimental induction of PMWS and PDNS in co-infection with TTSuV has shown that TTSuV1 might contribute to the disease expression (ELLIS; ALLAN; KRAKOWKA, 2008; KRAKOWKA et al., 2008). Studies have compared the TTSuV viral

loads between PCV2-negative and PCVAD-affected pigs and the results are controversial. A study has reported no significant TTSuV DNA loads differences between the two groups (LEE et al., 2010), while another study showed TTSuV2 loads significantly higher in PMWS-affected pigs (ARAMOUNI et al., 2011).

Lung tissues from animals presenting clinical signs of porcine respiratory disease complex (PRDC), a multifactorial disease syndrome involving porcine reproductive and respiratory syndrome virus (PRRSV), PCV2, *Mycoplasma hyopneumoniae*, and swine influenza virus (SIV), have been presented significantly higher co-infected with TTSuV1 than with TTSuV2, associating the virus species to the occurrence of the clinical PRDC (RAMMOHAN et al., 2012).

The TTSuV infection has also been evaluated in co-infection with hepatitis E virus (HEV), porcine reproductive and respiratory syndrome virus (PRRSV), and classical swine fever virus (CSFV). While the results of infectious hepatitis demonstrated an association between TTSuV, PCV2, and HEV, and indicated that TTSuV may play a role as a co-factor in the pathogenesis of the diseases (SAVIC et al., 2010), no correlation between TTSuV, PRRSV, and CSFV clinical signs or diseases has been identified (ZHU et al., 2012). However, the evaluation of the TTSuV DNA loads in experimentally CSFV-infected pigs has revealed that specifically the TTSuV2 serum load increased significantly in pigs with clinical signs of disease compared with the healthy animals also challenged (ARAMOUNI et al., 2013b).

TTSuV1 and TTSuV2 infections have been separately evaluated in gnotobiotic and specific pathogen free (SPF) pigs, and the presented lesions were associated to each of the virus species. TTSuV1 has been reported as the causative agent of mild respiratory lesions in gnotobiotic pigs (KRAKOWKA; ELLIS, 2008), while the TTSuV2 has been described to be directly related to parenchymatous organ lesions after the inoculation in SPF pigs (MEI et al., 2011). TTSuV2 has also been related to lesions of interstitial pneumonia and suggested to display a potential pathogenic effect in pigs (ARAMOUNI et al., 2013a).

Multiple infections with both TTSuV species and distinct TTSuV strains within the same species commonly occur (GALLEI et al., 2010; HUANG et al., 2010a). The simultaneous presence of several related but distinct TTSuV strains has been suggested to favor immune evasion by the virus, establishment of infection and, eventually, disease induction (GALLEI et al., 2010).

It has been suggested that, as in the *Circoviridae* family, in which PCV1 has been demonstrated to be non-pathogenic for pigs, while PCV2 has been certainly implicated

in disease occurrence, some anelloviruses (also a ssDNA virus family) might be linked to disease and TTSuV2 possible play a role in the development of multifactorial syndromes (ARAMOUNI et al., 2013b). This idea is suggestive supported by the TTSuV genetic variability, which could lead to differentiated biological behavior (CORTEY et al., 2011; ARAMOUNI et al., 2013b). Nevertheless, the implication of TTSuV in PCVADs and other viral pathologies remains to be elucidated.

An important consideration has been made by Meng (2012), who raised the possibility of the TTSuV infection generates adverse effects or interferes with the efficacies of common modified live-attenuated porcine vaccines. TTSuV1 has been suggested to have some immunosuppressive effect on the host immune response generated against PRRSV after vaccination (ZHANG et al., 2012). On the other hand, no differences have been observed in TTSuV2 DNA load and prevalence between PCV2-vaccinated and non-vaccinated pigs (NIETO et al., 2012).

### 1.3.5 – Immunogenicity

In 2011, the first serological assay for investigation of the TTSuV seroprevalence has been developed (HUANG et al., 2011).

After the expression and purification of the ORF1 capsid protein of TTSuV2, Western blot and indirect TTSuV2-specific ELISA assays were developed (HUANG et al., 2011). The results revealed the existence of anti-TTSuV2-ORF1 IgG antibodies in the pig serum evaluated and also a high rate of TTSuV2-seropositive pigs from conventional farms. It has been observed that PCVAD-affected pigs had a significantly lower level of TTSuV2 antibody than PCVAD-unaffected pigs, which could be due to the immunosuppression resulting of the PCV2 infection. A two-point longitudinal analysis of 2-month period showed an association between anti-ORF1 IgG antibody levels and the decrease of TTSuV2 viral loads or presumed virus clearance. The authors speculated that the production of ORF1-specific antibodies in response to the TTSuV2 infection may resolve the virus active infection *in vivo* over time.

In this same study, older pigs had higher seropositive rates and higher incidences of viremia, suggesting that age may be an important factor in regard TTSuV2 viremia and antibodies prevalence.

A second study group evaluated the TTSuV2 seroprevalence in domestic pigs of different ages in the United States by a modified version of the previously described

ELISA assay (XIAO et al., 2012). The results revealed a progressive increase of the anti-TTSuV2 antibody levels from very young to mature pigs, with the finisher pigs presenting the highest prevalence of TTSuV infection.

Although the importance of the serodiagnostic assay, this tool is so recent that there has been no time for it to be used on large scale and generate a large number of new data. Therefore, the prevalence of specific TTSuV antibodies is still unknown.

### 1.3.6 – Diagnosis

The diagnosis of TTSuV infection is currently based on nucleic acid detection (LEE et al., 2010). Serological assays, immunohistochemical, and *in situ* hibridization techniques, as well as viral culture system, are specific research tools that still have to be well established for this virus (KEKARAINEN; SEGALÉS, 2009).

The most used molecular tools available for detection of TTSuV DNA are the conventional PCR and nested-PCR (nPCR) (HUANG et al., 2010b). The development of real-time (or quantitative), SYBR green-based real-time, and duplex-PCR techniques, as well as the rolling circle amplification (RCA) assay, provided additional assays that may enhance the specificity, reduce time and labor of the TTSuV molecular diagnosis, in addition to be less expensive than conventional PCR method (BRASSARD et al., 2010; HUANG et al., 2010b; LEE et al., 2010; MACERA et al., 2011).

The Western blot and indirect ELISA assays to detect TTSuV2-specific antibodies in pig serum are the unique serodiagnostics and the most recent tools to evaluate the TTSuV-specific humoral immune responses (HUANG et al., 2011).

Histopathological studies have been described as another useful tool for the evaluation of TTSuV infection, although its shortage uses (MEI et al., 2011).

### 1.3.7 – TTSuV in Brazil

The first description of distinct species of TTSuV has been performed in Brazil (NIEL; DINIZ-MENDES; DEVALLE, 2005).

Reproductive organs, semen, ovarian follicular fluid, and lymph nodes of adult pigs have been evaluated for the presence of TTSuV in a study from Santa Catarina state (RITTERBUSCH et al., 2012), and they found TTSuV2 infection more frequently than TTSuV1 during co-infection with PCV2.

The evaluation of viremia and shedding of TTSuV and PCV2 has revealed that pigs at different ages from São Paulo state are commonly positive for both of the viruses in co-infection (DE CASTRO et al., 2012).

At present, a small number of studies evaluated the TTSuV infection in Brazilian pig herds. The lack of information regarding the TTSuV infection does not allow knowing the epidemiology of the virus in Brazil and, therefore, the implications of TTSuV in the pig health and productivity.

## 1.4 – CONCLUSION

A number of studies about TTV infection are available, but the biological properties of the virus are still unknown (HINO; MIYATA, 2007). The use of pigs as experimental model system to study transmission, infection dynamics, and tissue distribution of TTV infection in humans has been proposed (KEKARAINEN; SEGALÉS, 2009). The search for effective cell culture systems that support TTVs replication and the effort to better understand pathogenesis and immunogenicity of the virus must be continuous. In Brazil, the TTSuV epidemiological and molecular data are few. Further studies are necessary to improve the knowledge about the Brazilian distribution and phylogeny of pig aneloviruses.

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# 2 OBJETIVOS

## 2.1 – OBJETIVO GERAL

- Detectar e caracterizar molecularmente cepas de *torque teno sus virus* a partir de amostras biológicas de animais de diferentes categorias do ciclo de produção provenientes de rebanhos suinícolas brasileiros.

## 2.2 – OBJETIVOS ESPECÍFICOS

- Detectar por meio da PCR, com *primers* específicos para a UTR do genoma viral, a presença do TTSuV1 e TTSuVk2 em amostras de fezes de leitões de 1 a 3 semanas de idade provenientes das três principais regiões (Sul, Sudeste e Centro-oeste) produtoras de suínos do Brasil;
- Detectar por meio da PCR, com *primers* específicos para a UTR do genoma viral, a presença do TTSuV1 e TTSuVk2 em amostras de fezes de animais de diferentes categorias do ciclo de produção de suínos provenientes da região Oeste do estado do Paraná;
- Detectar por meio da PCR, com *primers* específicos para a UTR do genoma viral, a presença do TTSuV1 e TTSuVk2 em amostras pareadas de soro e fragmentos de tecidos (fígado ou pulmão) de suínos provenientes do estado do Paraná;
- Sequenciar os fragmentos de nucleotídeos amplificados para a identificação viral;
- Verificar a distribuição dos gêneros de TTSuV nas diferentes amostras biológicas e nas diferentes categorias do ciclo de produção de suínos;
- Realizar análise filogenética comparativa das cepas virais encontradas com as cepas virais de referência (protótipos) disponíveis em bases públicas de dados *(GenBank)*;
- Avaliar a ocorrência de infecção mista simultânea por cepas distintas dos gêneros de TTSuV nos mesmos animais.

## 2.3 – General Aim

- To detect and molecularly characterize strains of torque teno sus virus from different biological samples of animals of different stages of production cycle from Brazilian pig herds.

## 2.4 – Specific Aims

- To detect by means of PCR, with specific primers for the UTR of the viral genome, the presence of TTSuV1 and TTSuVk2 from fecal samples of piglets of 1 to 3 weeks of age from the three major pig-producing regions (South, Southeast, and Midwest) of Brazil;
- To detect by means of PCR, with specific primers for the UTR of the viral genome, the presence of TTSuV1 and TTSuVk2 from fecal samples of animals at different stages of pig production cycle of herds in the western region of Paraná state, Brazil;
- To detect by means of PCR, with specific primers for the UTR of the viral genome, the presence of TTSuV1 and TTSuVk2 from paired serum and organ (liver or lung) fragments of pigs of Paraná state, Brazil;
- To sequence the nucleotide fragments amplified;
- To verify the distribution of TTSuV genera in the different biological samples and in the different stages of pig production cycle;
- To perform comparative phylogenetic analyses of the detected viral strains with TTSuV prototypes available in public data bases (GenBank);
- To evaluate the ocurrence of simultaneous mixed infection by distinct strains of TTSuV genera in the same animals.

# 3 ARTIGOS PARA PUBLICAÇÃO

## 3.1 – **Infecção pelo *Torque teno sus virus* (TTSuV) em Leitões Lactentes de Rebanhos Suinícolas Brasileiros**

## ***Torque teno sus virus* (TTSuV) Infection in Suckling Piglets from Brazilian Pig Herds**

# *Torque teno sus virus infection in suckling piglets from Brazilian pig herds*

**Raquel de Arruda Leme, Alice Fernandes Alfieri & Amauri Alcindo Alfieri**

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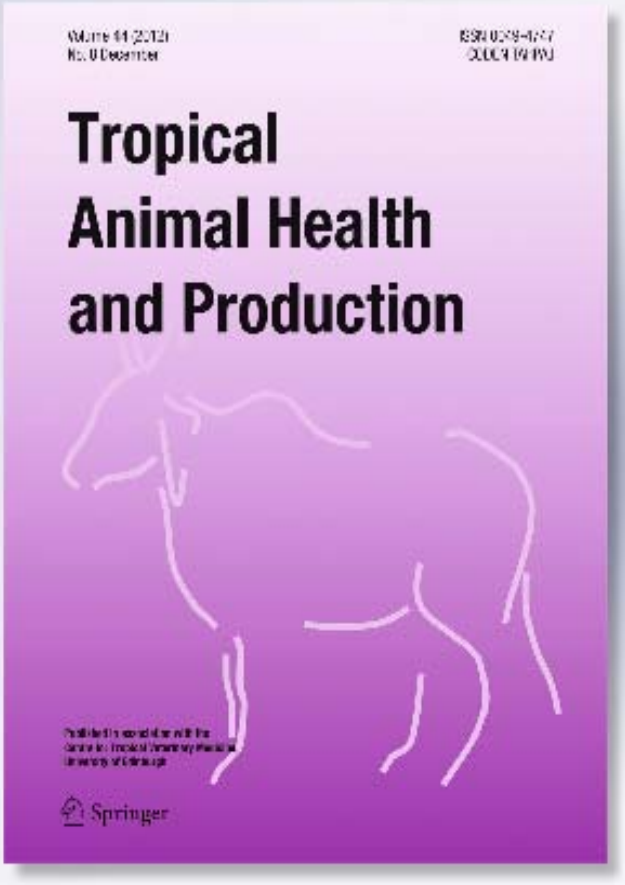


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ORIGINAL RESEARCH

# Torque teno sus virus infection in suckling piglets from Brazilian pig herds

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**Abstract** Torque teno sus virus (TTSuV) is responsible for the infection of pig herds around the world. The aim of this study was to analyse the presence of natural infection by both species of TTSuV in suckling piglets from major pig-producing regions of Brazil. Faecal samples ($n$=135) from 1 to 3-week-old suckling piglets from the Southern, Southeast and Midwest regions of Brazil were analysed by PCR assay to detect TTSuV1 and 2. TTSuV1 and 2 DNA was identified in 65 (48.1 %) and 23 (17 %) of piglet faecal samples, respectively. Co-infection by both species of TTSuV was detected in 17 (12.6 %) samples. Detection of TTSuV1 was significantly higher than that of TTSuV2 in the three Brazilian regions together ($p$<0.05). Based on age of animals, TTSuV1 infection was statistically higher than TTSuV2 in each age group ($p$<0.05). For all of the age groups together, no statistical difference was detected in the number of TTSuV1 and 2 positive results ($p$>0.05). These findings revealed that TTSuV infection has disseminated in pig herds from different geographic Brazilian regions, and the presence of TTSuV in suckling piglet faecal samples suggested the early infection by the virus and the potential of these animals in spreading the virus.

**Keywords** Swine · TTSuV1 · TTSuV2 · Faeces · Early infection · PCR

## Introduction

Torque teno virus (TTV) was first isolated from a Japanese man with non-A-E post-transfusion hepatitis. Although TTV has not been linked to any specific disease, its presence is being evaluated in many countries and in different animal species, mainly in human and swine. TTV, which belongs to the family *Anelloviridae*, is non-enveloped and has a negative-sense single-stranded circular DNA (ssDNA) genome (Kekarainen and Segalés 2012).

Pigs were first described as positive for Torque teno sus virus (TTSuV) by Leary et al. (1999) in the USA, and the entire genome sequence of the virus was first determined by Okamoto et al. (2002) in Japan. TTSuV is widespread in pig-producing regions of various countries, including Japan, China, Thailand, Serbia, Germany, Spain, Canada, USA, Cuba and Brazil (Gallei et al. 2010; Savic et al. 2010; Pérez et al. 2011; Meng 2012). The TTSuV genome has approximately 2.8 kb and the virus has been classified into two different genogroups, named genogroup 1 and genogroup 2 (Niel et al. 2005). In 2010, a new classification was established that designated the genogroups as two distinct species, torque teno sus virus 1 and torque teno sus virus 2, in the genus *Iotatorquevirus* and *Kappatorquevirus*, respectively, (ICTV 2011).

The prevalence of infection by TTSuV has been analysed in serum samples of pigs from different geographic regions of the world. The results have varied with between 24 and 100 % of animals determined to be positive for the virus (Kekarainen and Segalés 2012). The prevalence of TTSuV1 and 2 in serum, nasal secretions, stool, and organ samples have been demonstrated to increase with the age of the animals (Sibila et al. 2009b; Aramouni et al. 2010; Jarosova et al. 2011).

The TTSuV infection is common to both healthy and diseased pigs, and a number of studies have been performed to determine the importance of the virus and the role it plays in infectious diseases (Meng 2012). The TTSuV1 and 2 infections in gnotobiotic pigs have been separately evaluated, and the presented lesions were associated to the presence of each virus species (Krakowka and Ellis 2008; Mei et al.

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2011). The experimental induction of post-weaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS), both caused by porcine circovirus 2 (PCV2), in co-infection with TTSuV has shown that TTSuV1 might contribute to the disease expression (Ellis et al. 2008; Krakowka et al. 2008). Another study has compared the TTSuV viral loads between PCV2-negative pigs and PCV-associated disease (PCVAD)-affected pigs, and no statistically difference between the two groups was found (Lee et al. 2010).

The TTSuV infection also has been evaluated in co-infection with hepatitis E virus (HEV), porcine reproductive and respiratory syndrome (PRRS) and classical swine fever virus (CSFV). While the results of infectious hepatitis have demonstrated an association between TTSuV, PCV2 and HEV and indicated that TTSuV may play a role as a co-factor in the pathogenesis of the diseases (Savic et al. 2010), no correlation between TTSuV, PRRS and CSFV clinical signs or diseases has been identified (Zhu et al. 2012).

The detection of TTSuV infection is current based on conventional and real-time PCR assays. Recently, the first Western blot and indirect ELISA assays to detect TTSuV2-specific IgG antibodies in pig serum have been developed (Huang et al. 2011), and the shortage of TTSuV infection studies, which is based on the histopathological evaluation, has been presented (Mei et al. 2011). However, serological assays, immunohistochemical and in situ hybridization techniques, as well as viral culture system, are specific research tools that still have to be well established (Kekarainen and Segalés 2009).

In Brazil, in the last 5 years, the Southern, Southeast and Midwest regions have been the main producers of pigs. These three regions together were responsible for 92.9 % of the total industrial pig meat produced in 2010, in Brazil (ABIPECS 2011).

The presence of natural infection by TTSuV in suckling piglets from different regions of Brazil is still unknown. To determine the presence of TTSuV in the major pig-producing regions of Brazil is important, since there is a chance that the virus might compromise the health and productivity of pig herds and also be a co-factor for other diseases of viral aetiology that have economic impact. Suckling piglets were considered by our group as the starting point of this study, since it has been demonstrated that the prevalence increases throughout productive pig life. The aim of this study was to verify the occurrence of natural early TTSuV1 and 2 infections in piglets in herds from seven states of three different Brazilian regions.

## Materials and methods

All of the faecal samples were randomly selected, except for the state of origin and age of the animals, and analysed independently of other diagnostic results and the consistency of the faeces (diarrhoeic or not). First, porcine faecal samples from states within the three major pig-producing regions of Brazil were selected. Then, the faecal samples were chosen according to the age of the animals; only faecal samples from suckling piglets were selected.

One hundred and thirty-five faecal samples from piglets aged 1 to 3 weeks old were included in this study. The samples were derived from a collection of piglet faeces from 2004 to 2011 and were stored at 4°C.

Piglet faecal samples from each of the following Brazilian states were analysed: Rio Grande do Sul (RS, $n$=19), Santa Catarina (SC, $n$=18) and Parana (PR, $n$=16) from the Southern region; São Paulo (SP, $n$=35) and Minas Gerais (MG, $n$=10), representing the Southeast region; and Mato Grosso do Sul (MS, $n$=15) and Mato Grosso (MT, $n$=22) from the Brazilian Midwest region. When the faecal samples were from the same state, they were selected according to the city. Samples from different cities in the same state were selected. When samples were from the same city in the same state, they were selected by different farms where animals were raised. A total of 51 pig herds were included in this study.

Faecal suspensions were prepared at 10 to 20 % ($w/v$) in 0.01 M phosphate-buffered saline (PBS) and pH 7.2 and centrifuged at 5,000×$g$ for 3 min. The supernatants were used for DNA extraction.

To determine the occurrence of TTSuV infection, the viral ssDNA was extracted using a combination of phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate nucleic acid extraction methods (Alfieri et al. 2006) and immediately submitted to the polymerase chain reaction (PCR) assay.

Specific PCR assays were performed using primers for TTSuV1 and 2, targeting the non-coding region of the viral genome, and the technique was performed according to Segalés et al. (2009), with some modifications. In a final volume of 20 μl, 4 μl of the extracted DNA, 1× PCR buffer (20 mM Tris–HCl pH 8.4 and 50 mM KCl), 0.5 μl (20 pMol) of each primer, 1.25 mM total dNTP, 2 mM $MgCl_2$ and 0.75 U Platinum® Taq DNA polymerase (Invitrogen™ Life Technologies, São Paulo, Brazil) were added.

Serum samples previously known to be positive for TTSuV1 and TTSuV2 by the PCR assay and confirmed by sequencing analyses were used as positive controls. Sterile ultrapure water was used as the negative control. The amplification reaction was performed in a thermocycler (Swift™ MaxPro Thermal Cycler, Esco Healthcare Pte, Singapore) at 94°C for 5 min for denaturation followed by 40 cycles of 94, 54 and 72°C/min and a final extension at 72°C for 5 min.

The amplified products were analysed by electrophoresis on a 1 % agarose gel in TBE buffer, pH 8.4 (89 mM Tris;

89 mM boric acid; 2 mM EDTA), stained with ethidium bromide (0.5 g/ml) and visualised under UV light. The expected sizes of the amplified products were 305 and 252 bp for TTSuV1 and 2, respectively.

To confirm the specificity of the amplicons obtained in this study, two positive samples for TTSuV1 and 2 were randomly selected for sequencing analyses. The amplicons were purified by QIAquick PCR purification Kit (Qiagen, Valencia, CA, USA), quantified with Qubit™ fluorometer (Invitrogen™ Life Technologies, Eugene, OR, USA) and analysed by electrophoresis on a 2 % agarose gel. An ABI3500 Genetic Analyser and the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) were used for sequencing, which was performed in both directions with forward and reverse primers. Sequence quality analyses were performed using Phred and CAP3 software (http://asparagin.cenargen.embrapa.br/phph/). Similarity searches were performed with sequences deposited in GenBank using the basic local alignment search tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree building based on nucleotide (nt) was obtained using the neighbour-joining algorithm based on Kimura two-parameter model, which provided statistical support via bootstrapping with 1,000 replicates using the MEGA package (version 4.0). Sequence identity matrix was performed using the BioEdit software version 7.0.8.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

The Chi-square statistical test was performed to compare the frequencies of TTSuV1 and 2 in the regions evaluated (Epi Info™ 6.04d). For the TTSuV infection by age, statistical analyses were performed using the non-parametric Kruskal–Wallis test, and when significant differences were present, the data were submitted to the Dunn test (Biostat 5.0). The confidence limit for the statistical tests was set at 95 % ($p<0.05$).

## Results

TTSuV was detected in pig herds from all three of the Brazilian regions evaluated in this study. Among the 135 piglet faecal samples analysed, infection by TTSuV1 and 2 was found in 65 (48.1 %) and 23 (17 %) of the suckling piglets, respectively. This difference in the positive infection results for each species of TTSuV was statistically significant ($p=0.001$) when the three regions were analysed together. The results of TTSuV infection in pig herds from the three Brazilian regions are represented in Table 1.

Mixed infection by both species of TTSuV was found in all of the seven states evaluated. Among the 135 piglet faecal samples, co-infection was found in 17 (12.6 %) samples.

To analyse TTSuV infection at the different ages, the piglet faecal samples were divided according to the age of the animals into groups of 1 ($n=42$), 2 ($n=44$) and 3 ($n=49$) weeks old animals. The percentage of TTSuV1 and TTSuV2 positive results in the suckling piglet faecal samples, according to the age of the animals, are represented in Fig. 1. TTSuV1 was detected at a statistically significant higher frequency than TTSuV2 in each age group ($p=0.0225$, $p=0.0246$ and $p=0.0014$, respectively) (Table 2). However, a comparison of infection by TTSuV1 among the three age groups indicated no statistical difference in the detection of TTSuV1 ($p=0.6726$). Similar results were obtained for the detection of TTSuV2 infection ($p=0.8797$). Moreover, mixed infection by both species of TTSuV was observed in all three age groups.

The nucleotide sequences of the TTSuV1 (TTSuV1_BRA11/07) and 2 (TTSuV2_BRA21/11) amplicons obtained in this study contain 304 and 224 bp, respectively. A comparison of these sequences to those available in GenBank

**Table 1** Detection of TTSuV1 and TTSuV2 DNA in suckling piglet faecal samples from Brazilian pig herds by PCR assay

| Region | Number of samples evaluated | Positive samples | |
|---|---|---|---|
| | | TTSuV1 (%) | TTSuV2 (%) |
| Southern[a] | 53 | 27 (50.9) | 10 (18.9) |
| Southeast[b] | 45 | 23 (51.1) | 4 (8.8) |
| Midwest[c] | 37 | 15 (40.5) | 9 (24.3) |
| Total | 135 | 65* (48.1) | 23* (17) |

*$p<0.05$, statistically significant differences

[a] RS ($n=19$), SC ($n=18$) and PR ($n=16$)

[b] MG ($n=10$) and SP ($n=35$)

[c] MS ($n=15$) and MT ($n=22$)

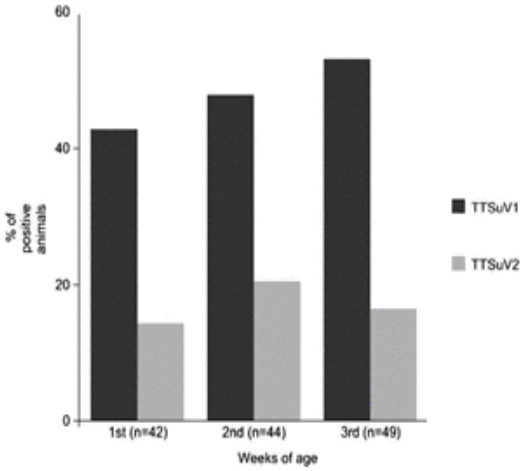


**Fig. 1** Percentage of TTSuV1 and TTSuV2 positive results in the suckling piglet faecal samples according to the age of the animals

**Table 2** Comparison of TTSuV1 and TTSuV2 infection between the different age groups by Kruskal–Wallis and Dunn statistic tests

| Statistical data | Piglet age group (weeks) | | |
|---|---|---|---|
| | First ($n$=42) | Second ($n$=44) | Third ($n$=49) |
| TTSuV1-positive samples (mean) | 18 (0.42[a]) | 21 (0.48[a]) | 26 (0.53[a]) |
| TTSuV2-positive samples (mean) | 6 (0.14[b]) | 9 (0.20[b]) | 8 (0.16[b]) |
| $H$ | 5.2035 | 5.054 | 10.2457 |
| $p$ (Kruskal–Wallis) | 0.0225 | 0.0246 | 0.0014 |
| TTSuV1 rank sum (medium rank) | 2,037 (48.5) | 2,222 (50.5) | 2,866.5 (58.5) |
| TTSuV2 rank sum (medium rank) | 1,533 (36.5) | 1,694 (38.5) | 1,984.5 (40.5) |
| Ranks difference* | 12 | 12 | 18 |
| $Z$ calc* | 2.2544 | 2.2032 | 3.1334 |

Values with letters indicate statistically significant differences ($p$<0.05)

*$p$<0.05, comparisons by the Dunn test ($Z$ critical=1.96)

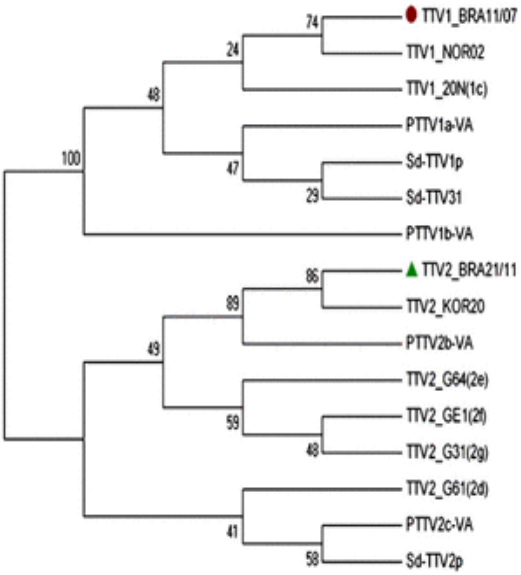


**Fig. 2** Neighbour-joining phylogenetic tree (Kimura two-parameter model) with 269 nt (20–288), which was reconstructed based on TTSuV1 and 2 nucleotide sequences in this work, presented TTSuV1_BRA11/07(JQ619841) (*red-filled circle*) and TTSuV2_-BRA21/11(JQ619842) (*green-filled triangle*) and others acquired in GenBank: Sd-TTV31 (AB076001), Sd-TTV1p (AY823990), Sd-TTV2p (AY823991), PTTV1a-VA (GU456383), PTTV1b-VA (GU456384), TTV1_20N (GU570199), PTTV2b-VA (GU456385), PTTV2c-VA (GU456386), TTV2_G64 (GU570208), TTV2_GE1 (GU570209), TTV2_G31 (GU570204), TTV2_G61 (GU570207), TTV1_NOR02 (JF451488) and TTV2_KOR20 (JF451601). The *major upper* and *lower branches* refer to TTSuV1 and TTSuV2, respectively

indicated that TTSuV1 shows 98 % nt similarity to a strain from Norway (TTV1_NOR02), while TTSuV2 shows 100 % nt similarity to a strain from Korea (TTV2_KOR20). The TTSuV1 and 2 sequences in this study were also compared to other nt sequences that serve as prototypes for studies (Sd-TTV31, Sd-TTV1p and Sd-TTV2p) and have been cited in other works that suggest the classification of TTSuV strains into types and subtypes within the species (PTTV1a-VA, PTTV1b-VA, TTV1_20N, PTTV2b-VA, PTTV2c-VA, TTV2_G64, TTV2_GE1, TTV2_G31 and TTV2_G61). The phylogenetic tree with a presentation of the results is represented in Fig. 2.

## Discussion

The three regions represented in this study are responsible for the major production of pork meat in Brazil. TTSuV infection was present in all of these regions, suggesting that the virus is widespread in Brazilian pig herds. Because the presence of TTSuV1 was statistically higher than TTSuV2 in these regions, the TTSuV1 species can be assumed to more frequently infect suckling piglets from Brazil. Moreover, the high frequency of natural infection by TTSuV in suckling piglets showed that these animals had early contact with the virus, which raises questions regarding the routes of transmission of the virus.

Sibila et al. (2009a), in Spain, have studied the infection by both species of TTSuV in sows and piglets and found that TTSuV1 is present at a higher frequency than TTSuV2. Pérez et al. (2011), in Cuba, have shown that infection by TTSuV1 is more frequent than infection by TTSuV2, even when animals are co-infected with other viral pathogens. In contrast, Segalés et al. (2009), in Spain, have determined that TTSuV2 was more prevalent than TTSuV1 in a 20-year retrospective study.

In Brazil, Niel et al. (2005) detected for the first time the presence of distinct species of TTSuV. Ritterbusch et al. (2011), in Santa Catarina state, Brazil, have described the presence of TTSuV1 and 2 in the swine reproductive tract in co-infection with PCV2 and have shown a higher prevalence of TTSuV2, primarily in co-infections with PCV2. Beyond these studies, little is known regarding the natural infection by TTSuV in Brazil.

The route of TTSuV transmission has been demonstrated to vary, and all of the routes are epidemiologically important. Kekarainen et al. (2007) have demonstrated the presence of TTSuV in boar semen and suggested the venereal route as an important route of infection. Pozzuto et al. (2009) have shown that TTSuV may be transmitted to piglets by an in utero route. Sibila et al. (2009a) have found TTSuV infection in sows and piglets, reinforcing the possibility that vertical transmission may occur and that piglet-to-piglet transmission is also important. Sibila et al. (2009b)

have also demonstrated the presence of the virus in nasal swabs, which suggests that oro-nasal is another transmission route. Brassard et al. (2008) have detected the TTSuV in pig faecal samples and not only suggested that the virus may be transmitted by a faecal-oral route but also indicated that some farming practices, such as the application of pig manure and irrigation with river water, can be responsible for the introduction and permanence of TTSuV in the environment.

In our study, the frequency of mixed infection by both species of TTSuV (12.6 %) showed that co-infection of piglets was not unusual. However, understanding what these data mean is still not possible because it is not known whether the presence of one of the species limits or favours infection by the other. Moreover, co-infection may also interfere in viral shedding, whether the both species of TTSuV act in a competitive or synergistic way is also unknown. Therefore, the importance of co-infection by both species of TTSuV is still unknown.

Sibila et al. (2009a) have examined the number of positive TTSuV sows that delivered stillborn piglets and shown that a significantly higher number of sows that were co-infected by both species of TTSuV deliver stillborn piglets compared with the non-co-infected sows. At that time, although a small number of samples were analysed, both species of TTSuV were suggested to potentially play an important role in reproductive failure.

This study also intended to evaluate the frequency of infection by TTSuV in suckling piglets of different ages, and the results presented here showed that in all of the age groups TTSuV1 was significantly more frequently detected than TTSuV2 in suckling piglets. The results presented by this study indicated that discretely higher percentage of 3-week-old animals were positive for TTSuV compared with animals at 1 week of age. Although this difference is mild and not significant ($p>0.05$), it may suggest that infection increases with the age of the animals.

The faecal samples analysed in this study were collected between 2004 and 2011, and positive results for TTSuV1 and 2 or co-infection with both species could be observed throughout this time interval. Therefore, this data represent a retrospective study of 7 years and show that the presence of the virus in pig herds in Brazil is not a recent development. Segalés et al. (2009), in Spain, showed the earliest evidence of TTSuV infection, 14 years before the first description of the virus.

Few Brazilian TTSuV sequences are available on GenBank. The results presented here provide some phylogenetic information for the TTSuV strains from Brazil and may serve as an additional tool for future studies.

To the best of our knowledge, this unique study represents the first description of TTSuV infection in suckling piglets from states that represent the major pig-producing regions of Brazil. The results presented here show that infection by TTSuV has disseminated in Brazilian pig herds for years (2004–2011). The presence of TTSuV in suckling piglet faecal samples revealed the early infection and the capacity of these animals in disseminating the virus, even during the suckling age. Other studies that aim to better understand the behaviour of the virus may help to elucidate the TTSuV epidemiology, evolution, potential of infection and pathogenicity, primarily in co-infection with emerging viral diseases.

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## 3.2 – **Infecção pelo *Torque teno sus virus* (TTSuV) em Diferentes Categorias do Ciclo de Produção de Suínos**

## ***Torque teno sus virus* (TTSuV) Infection at Different Stages of Pig Production Cycle**[1]

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LEME, R. A.; ALFIERI, A. F.; ALFIERI, A. A. 2013 ***Torque teno sus virus* (TTSuV) infection at different stages of pig production cycle**. *Pesquisa Veterinária Brasileira* 00(0): 00-00. Laboratory of Animal Virology, Department of Veterinary Preventive Medicine, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid - Campus Universitário. 86057-970 - Londrina – PR, Brazil PO Box 10011. E-mail: alfieri@uel.br

**Abstract**: *Torque teno sus virus* (TTSuV) infection is present in pig herds worldwide. It has been demonstrated that TTSuV might increase the severity of other important viral diseases with economic and public health impacts. At present, there is no information on the age distribution of pigs infected with TTSuV in Brazilian herds. This study evaluated the frequency of TTSuV infection in pigs at different stages of production. Fecal samples (*n*=190) from pigs at 1 to 24 weeks of age and from breeders at 6 farrow-to-weaning and 9 grower-to-finish farms in the western region of Paraná state, Brazil, were evaluated by PCR. Fragments of the 5' UTRs of TTSuV1 and/or TTSuVk2 DNAs were identified in 126 (66.3%) of the fecal samples. All the evaluated pig production stages presented positive results for TTSuV infection. Significant differences were found with the percentages of positive samples for TTSuV1, TTSuVk2, and mixed infections by both genera between and within the stages. TTSuV detection was significantly more frequent in finisher pigs ($p<0.05$) than in the animals from the other stages. TTSuV1 and TTSuVk2-induced infections were more frequently observed in suckling piglets and finisher pigs, respectively. These results suggest that TTSuV infection has spread to pigs of all production stages and that the viral infection rate increases with the age of the animals in herds from the western region of Paraná state, Brazil.

**Index Terms**: Porcine. *Iotatorquevirus*. *Kappatorquevirus*. TTSuV1 and TTSuVk2. Age distribution. PCR.

**Resumo**: [**Infecção pelo *Torque teno sus virus* (TTSuV) em diferentes categorias do ciclo de produção de suínos.]** A infecção pelo *Torque teno sus virus* (TTSuV) está presente em rebanhos suinícolas em todo o mundo. Tem sido demonstrado que a infecção pelo TTSuV pode aumentar a gravidade de outras importantes doenças virais com impactos econômico e na saúde pública. Atualmente não há informações sobre a distribuição da infecção pelo TTSuV, de acordo com a faixa etária, em rebanhos suinícolas brasileiros. Este estudo avaliou a frequência da infecção pelo TTSuV nas diferentes categorias de produção de suínos. Amostras fecais (*n*=190) de suínos com 1 a 24 semanas de idade e de reprodutores,

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provenientes 6 unidades produtoras de leitões e 9 unidades de terminação da região oeste do Paraná, Brasil, foram avaliadas pela técnica de PCR. Fragmentos da região 5' UTR do DNA do TTSuV1 e TTSuVk2 foram identificados em 126 (66,3%) amostras fecais. Todas as categorias de produção avaliadas apresentaram resultados positivos para a infecção pelo TTSuV. Diferenças significativas foram encontradas em relação às porcentagens de amostras positivas para o TTSuV1, TTSuVk2 e infecção mista de ambos os gêneros inter e intra categorias. A detecção do TTSuV em animais de terminação foi significativamente mais frequente ($p<0.05$) do que nos suínos de outras categorias. Infecções induzidas pelo TTSuV1 e TTSuVk2 foram mais frequentemente observadas em leitões de maternidade e suínos de terminação, respectivamente. Estes resultados sugerem que a infecção pelo TTSuV encontra-se disseminada em suínos de todas as categorias de produção e que a taxa da infecção viral aumenta de acordo com a idade dos animais em rebanhos da região oeste do estado do Paraná.

**Termos de Indexação**: Suínos. *Iotatorquevirus*. *Kappatorquevirus*. TTSuV1 e TTSuVk2. Distribuição por idade. PCR.

## Introduction

*Torque teno virus* (TTV) is a non-enveloped virus with a circular negative-sense single-stranded DNA (ssDNA) genome. This virus was first isolated from a Japanese man with non A-E post-transfusional hepatitis (Nishizawa et al. 1997). Since then, TTV has been shown to infect humans, non-human primates, and farm animals (Leary et al. 1999, Okamoto et al. 2000, 2001, 2002, Brassard et al. 2008).

*Torque teno sus virus* (TTSuV) infection in pigs was first described by Leary et al. (1999). The first full genome analysis of TTSuV was completed by Okamoto et al. (2002), and the differentiation of TTSuV into separate species was performed by Niel, Diniz-Mendes & Devalle (2005). TTSuV belongs to the family *Anelloviridae* and is classified into the species TTSuV1a and TTSuV1b in the genus *Iotatorquevirus* and TTSuVk2 in the genus *Kappatorquevirus* (ICTV 2012).

TTSuV is widely distributed in pig herds in a number of countries in Asia, Europe, and America (McKeown et al. 2004, Niel, Diniz-Mendes & Devalle 2005, Gallei et al. 2010, Savic et al. 2010, Pérez et al. 2011, de Arruda Leme, Alfieri & Alfieri 2012). TTSuV infection is found in both healthy and diseased pigs, and a number of studies have been performed to determine the importance of the virus and the role it plays in infectious diseases (Meng 2012).

The detection of TTSuV infection is currently based on conventional and real-time PCR assays. Recently, the first Western blot and indirect ELISA assays to detect TTSuV2-specific IgG antibodies in pig serum were developed (Huang et al. 2011). The shortage of TTSuV infection studies based on histopathological technique has been discussed

(Mei et al. 2011). However, serological assays, immunohistochemical and *in situ* hybridization techniques, and viral culture systems are specific research tools that are not well established for this virus (Kekarainen & Segalés 2009, de Arruda Leme, Alfieri & Alfieri 2012).

Studies have demonstrated the presence of TTSuV in the serum and organs of pigs of different ages (Kekarainen & Segales 2012) and have shown that the prevalence increases with the age of the animals (Sibila et al. 2009b, Aramouni et al. 2010). In Brazil, TTSuV infection has been identified in suckling piglets (de Arruda Leme, Alfieri & Alfieri 2012) and slaughter-age pigs (de Arruda Leme et al. 2013), and in the reproductive tracts of boars and sows (Ritterbusch et al. 2012). Despite these studies, no information is available relative to the age distribution of TTSuV infection throughout the Brazilian pig production system.

The aims of this study were to evaluate natural infection by TTSuV in 1 to 24 week-old pigs and in breeders, and to evaluate the frequency of TTSuV infection at different stages of pig production.

## Materials and Methods

One hundred and ninety fecal samples of pigs from the western region of Paraná state, Brazil, were included in this study. The samples were selected independent of their consistency (diarrheic or not), collected between 2008 and 2011, and stored at 4°C. Six farrow-to-weaning farms, which include breeder sows and boars, suckling piglets, and weaned pigs up to 8 weeks of age, and nine grower-to-finish farms, where 9-week-old pigs were housed and fed until they reach 24 weeks of age, were evaluated. A total of 119 fecal samples from farrow-to-weaning farms and 71 samples from grower-to-finish farms were selected.

Fecal samples for each of the pig production stages were analyzed: suckling piglets (1 to 3 weeks old, $n$=35), weaned piglets (4 to 8 weeks old, $n$=43), finisher pigs (9 to 24 weeks old, $n$=71), and breeders ($n$=41).

Fecal suspensions were prepared at 10 to 20% (w/v) in 0.01 M phosphate-buffered saline (PBS), pH 7.2, and centrifuged at 5,000 x *g* for 3 min. The supernatants were used for DNA extraction.

To determine the frequency of TTSuV infection, viral ssDNA was extracted by using a combination of the phenol/chloroform/isoamyl alcohol (25:24:1) and

silica/guanidinium isothiocyanate nucleic acid extraction methods (Alfieri et al. 2006) and was immediately submitted to a polymerase chain reaction (PCR) assay.

Specific PCR assays were performed using primers for TTSuV1 (*Iotatorquevirus*) and TTSuVk2 (*Kappatorquevirus*) targeting the non-coding region of the viral genome, and the technique was performed as described (Segalés et al. 2009), with modifications (de Arruda Leme, Alfieri & Alfieri 2012). The amplification reaction was performed in a thermocycler (Swift™ MaxPro Thermal Cycler, Esco Healthcare Pte, Singapore) at 94°C for 5 min for denaturation followed by 40 cycles of 94°C/1 min, 54°C/1 min, and 72°C/1 min and a final extension at 72°C for 5 min. The expected sizes of the amplified products were 305 and 252 bp for TTSuV1 and k2, respectively.

Samples positive for TTSuV1 and TTSuVk2 were randomly selected for sequence analysis to confirm the specificity of the amplicons obtained in this study. The amplicons were purified by using a QIAquick PCR purification Kit (Qiagen, Valencia, CA, USA), quantified with a Qubit™ Fluorometer (Invitrogen™ Life Technologies, Eugene, OR, USA), and analyzed by electrophoresis on a 2% agarose gel. An ABI3500 Genetic Analyzer and the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) were used for sequencing, with forward and reverse primers. Sequence quality analysis was performed using Phred and CAP3 software (http://asparagin.cenargen.embrapa.br/phph/). Similarity searches were done with sequences deposited in GenBank by using the Basic Local Alignment Search Tool - BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis was performed with Epi Info™, using Chi-square ($\chi^2$) or Fisher exact tests to compare the proportions of TTSuV-positive samples between and within the pig production stages and to compare the detection rates of TTSuV of the different farm types (farrow-to-weaning vs. grower-to-finish). The confidence limit for the statistical tests was set at 95% ($p<0.05$).

## Results

Of the 190 pig fecal samples evaluated, 126 (66.3%) were positive for TTSuV infection. The TTSuV presence was detected in pigs of all stages evaluated. The specificity of the amplicons obtained for each TTSuV genus was confirmed during the sequence analysis. The TTSuV detection results for each stage of pig producing cycle are presented in Table 1.

**Table 1** – Detection rates of TTSuV1 and TTSuVk2 in single and mixed-infections by PCR assay in pig fecal samples according to stage of pig production cycle

| TTSuV detection | Suckling piglets (1 to 3 weeks) *n*=35 (%) | Weaned piglets (4 to 8 weeks) *n*=43 (%) | Finisher pigs (9 to 24 weeks) *n*=71 (%) | Breeders (♂=10/♀=31) *n*=41 (%) | Total *n*=190 |
|---|---|---|---|---|---|
| TTSuV1 | 10 (28.6) $^{A, a}$ | 10 (23.3) $^{A, a}$ | 4 (5.6) $^{B, a}$ | 8 (19.5) $^{A, a}$ | 32 (16.8) |
| TTSuVk2 | 3 (8.6) $^{A, b}$ | 12 (27.9) $^{C, a}$ | 39 (54.9) $^{B, b}$ | 5 (12.2) $^{A, C, a}$ | 59 (31) |
| TTSuV1 + k2 | 3 (8.6) $^{A, b}$ | 8 (18.6) $^{A, B, a}$ | 21 (29.5) $^{B, c}$ | 3 (7.3) $^{A, a}$ | 35 (18.4) |
| Total Positive | 16 (45.7) | 30 (69.8) | 64 (90.1) | 16 (39) | 126 (66.3) |
| Total Negative | 19 (54.3) | 13 (30.2) | 7 (9.9) | 25 (61) | 64 (33.7) |

Capital letters (A, B, and C) refer to comparisons between stages and lower case letters (a, b, and c) refer to comparisons within each of the stages. Different letters indicate statistically significant differences ($p<0.05$).

Among the pig production stages, single infections of TTSuV1 were significantly ($p<0.05$) less frequent in finisher pigs than in animals of other stages. The rate of single infections of TTSuVk2 was significantly ($p<0.05$) higher in finisher pigs relative to animals of other stages. A significantly ($p<0.05$) higher frequency of mixed infections due to TTSuV1 and TTSuVk2 was found in finisher pigs relative to suckling piglets and breeders.

When the proportions of TTSuV1- and TTSuVk2-positive samples within each stage were compared, no significant ($p>0.05$) differences were found for weaned piglets or breeders. However, the detection of TTSuV1 in suckling piglets was significantly ($p<0.05$) more frequent than the detection of TTSuVk2 or mixed infections of both genera. When the results of finisher pigs were evaluated, TTSuVk2 detection was significantly ($p<0.05$) more frequent than the detection of TTSuV1 or mixed infections by both genera. Mixed infections by TTSuV1 and TTSuVk2 were detected at significantly ($p<0.05$) higher rates than single infections of TTSuV1.

All farms evaluated had positive results for both TTSuV genera. The rates of TTSuV detection were 52.1% (62/119) and 90.1% (64/71) for samples from farrow-to-weaning and grower-to-finish farms, respectively, and the difference between these rates was significant ($p<0.05$). TTSuV1 and TTSuVk2 were detected in both single and mixed infections at all farrow-to-weaning farms (*n*=6); for the single infections of TTSuV1 were detected at 4 of grower-to-finish farms (*n*=9), whereas single infections of TTSuVk2 and mixed infections of TTSuV1+TTSuVk2 were observed in all farms evaluated (Table 2).

Positive results for TTSuV1, TTSuVk2, and mixed infections with both genera were observed throughout the period evaluated (2008-2011).

**Table 2** – TTSuV1 and TTSuVk2 single and mixed-infections in pig fecal samples according to farm type

| Farms | Pig herds (number of samples evaluated) | TTSuV detection | | | TOTAL (%) |
|---|---|---|---|---|---|
| | | TTSuV1 | TTSuVk2 | TTSuV1 + TTSuVk2 | |
| Farrow-to-weaning (breeder sows and boars, suckling piglets, and weaned pigs until 8 weeks of age) *n*=6 | FW I (*n*=19) | 6 | 2 | 3 | 11 (57.9) |
| | FW II (*n*=24) | 6 | 1 | 3 | 10 (41.7) |
| | FW III (*n*=22) | 6 | 4 | 1 | 11 (50) |
| | FW IV (*n*=16) | 1 | 8 | 1 | 10 (62.5) |
| | FW V (*n*=18) | 2 | 3 | 4 | 9 (50) |
| | FW VI (*n*=20) | 7 | 2 | 2 | 11 (55) |
| Subtotal | *n*=119 | 28 (23.5%) | 20 (16.8%) | 14 (11.8%) | 62 (52.1)[a] |
| Grower-to-finish (9 to 24 weeks of age) *n*=9 | GF I (*n*=7) | - | 4 | 3 | 7 (100) |
| | GF II (*n*=7) | - | 3 | 3 | 6 (85.7) |
| | GF III (*n*=9) | 1 | 5 | 1 | 7 (77.8) |
| | GF IV (*n*=5) | 1 | 2 | 2 | 5 (100) |
| | GF V (*n*=6) | 1 | 1 | 2 | 4 (66.7) |
| | GF VI (*n*=10) | - | 7 | 3 | 10 (100) |
| | GF VII (*n*=6) | - | 3 | 2 | 5 (83.3) |
| | GF VIII (*n*=4) | 1 | 1 | 2 | 4 (100) |
| | GF IX (*n*=17) | - | 13 | 3 | 16 (94.1) |
| Subtotal | *n*=71 | 4 (5.6%) | 39 (54.9%) | 21 (29.6%) | 64 (90.1)[b] |
| TOTAL | *n*=190 | 32 | 59 | 35 | 126 (66.3) |

Different letters indicate statistically significant differences ($p<0.05$).

## Discussion

As far as the authors are aware, this is first study that investigated the rates of TTSuV detection at different stages of pig production in Brazil. These results indicate that TTSuV infection has spread to pigs of all production stages. Finisher pigs had significantly

higher rates of TTSuV detection than animals from the other stages, in agreement with previous studies that have reported that TTSuV infection increases with the age of the animals (Sibila et al. 2009b, Aramouni et al. 2010).

In our study, the overall percentage of TTSuV-positive pigs (66.3%) was higher than that described in a study performed with rectal swabs (Sibila et al. 2009b), which reported a low percentage (<20%) of TTSuV detection. Previous studies reported higher rates for TTSuV in fecal samples, suggesting that the fecal-oral route is an important route of TTSuV transmission (Brassard et al. 2008, de Arruda Leme, Alfieri & Alfieri 2012).

An inversion in the frequencies of infection with the two TTSuV genera relative to the age of pigs within the production cycle was observed during this study. TTSuV1 detection was higher ($p<0.05$) in suckling piglets and progressively less frequent with the increasing age up to finisher pigs, whereas the detection rates of single infections of TTSuVk2 and mixed infections increased proportionally with the age of the animals; with finisher pigs presenting higher ($p<0.05$) rates of TTSuVk2 infection than the animals of the other stages. These findings are corroborated by the results at the farm level; 5 of the 9 grower-to-finish farms did not present positive results for single infections of TTSuV1. A similar result was obtained in a longitudinal study performed with 1 to 15 week-old animals, which evaluated the presence of TTSuV in different biological samples (Sibila et al. 2009b). Specifically, in that study TTSuV1 was detected more frequently than TTSuV2 from the nasal swabs of animals up to 7 weeks of age, but the rate of TTSuV2 detection was higher ($p<0.05$) than the rate of TTSuV1 in pigs that were at 11 and 15 weeks of age. Moreover, analysis of the rectal swabs demonstrated that the percentage of TTSuV2-positive pigs increased progressively from 7 weeks of age onwards, whereas the detection of TTSuV1-positive pigs was fairly constant throughout the study (Sibila et al. 2009b). Although the data from both studies show similar trends, there is no clear explanation for the inversion in the rates of infection by TTSuV species.

The prevalence of TTSuV infection in pigs of different ages has been evaluated in the United States and it was demonstrated that both TTSuV1 and TTSuV2 infections increased with the age of the animals, with the prevalence of TTSuV1 DNA being higher ($p<0.05$) than that of TTSuV2 in each of the age groups analyzed (Xiao et al. 2012). Although a serodiagnostic technique was used in the American study, the different geographical regions used in these two studies might explain this divergence in the results obtained, suggesting that the behavior of the virus may vary between distinct geographical locations.

It has been demonstrated that a single pig can be infected with more than one strain of the TTSuV genera (Huang et al. 2010, Gallei et al. 2010, de Arruda Leme et al. 2013). This is probably because molecular variation in the TTSuV genomic sequences could lead to antigenic differences as a mechanism to avoid the host immune response.

An increasing level of anti-TTSuV2-ORF1 antibodies has been shown to be associated with a decrease in the viral DNA load within the serum (Huang et al. 2011). Although the result refers to TTSuVk2, and considering the previous results that TTSuV1 species more frequently infects suckling piglets from Brazil (de Arruda Leme, Alfieri & Alfieri 2012), the progressive production of anti-TTSuV1 antibodies in response to early exposure is likely. Such antibody production might be at least partially responsible for decreases in virus excretion via feces and in the frequency of positive results for this species of the virus in older animals, since piglets are typically exposed to TTSuV1 earlier in life. However, the immunological response could not lead to the clearance of TTSuV1 infection, and the increasing incidence of infection by TTSuVk2 with age might also explain the increase in co-infection rates.

Age has been suggested to be an important factor affecting the profile of TTSuV2 infection because older pigs have been found to have higher prevalences of viremia and antibodies (Huang et al. 2011). The slow development of immunity as the animals become exposed and matures, suggested by Xiao et al. (2012), and the progressive increase in the frequency of TTSuVk2 infection with age could explain the high frequency of TTSuVk2-positive animals of this study.

TTSuV2-induced infections have been demonstrated to be more frequent in porcine circovirus associated disease (PCVAD)-affected pigs than in non-affected pigs (Kekarainen, Sibila & Segalés 2006, Blomström et al. 2010, Aramouni et al. 2011), although no association between TTSuV species infection and PCVAD has been identified (Lee et al. 2010). In Brazil, Ritterbusch et al. (2012) found TTSuV2 infection more frequently than TTSuV1 infection in the reproductive organs, semen, ovarian follicular fluid and lymph nodes of adult pigs, primarily in the context of co-infection with porcine circovirus 2 (PCV2).

In the present study, 18.4% (35/190) of the fecal samples were positive for both genera of TTSuV. A study performed by our group evaluated paired organs and serum samples from clinically healthy slaughter-age pigs for TTSuV infection, where it was demonstrated co-infection due to both TTSuV genera in 87.9% (102/116) of the samples, whereas single infections of TTSuV1 and TTSuVk2 were found in only 2 and 9 of the 116 samples analyzed, respectively (de Arruda Leme et al. 2013). The difference in the detection

rate of TTSuV-induced mixed infections between our two studies may be due to the type of sample evaluated (fecal vs. serum vs. organs). Based on the results obtained from the fecal samples, it can be suggested that mixed infections due to TTSuV genera may interfere with viral shedding, leading to lower rates of detection in feces. In the study based on the systemic infection (de Arruda Leme et al., 2013) it was not possible to determine whether action of the two genera of TTSuV is competitive or synergetic. Thus, it is clear that TTSuV genera co-infection is not an unusual event, but understanding the implications of this result has not been elucidated since it is not known whether the presence of one of the genus limits or favors infection by the other or affects the level of viral shedding (de Arruda Leme, Alfieri & Alfieri 2012).

In this study, the frequency of TTSuV infection for breeders was not significantly ($p>0.05$) different for that of suckling piglets. The results are in agreement with the detection of TTSuV in sows (Sibila et al. 2009a) but differ from the results obtained from wild boar serum samples, which showed that TTSuV2 infection was more common ($p<0.05$) than TTSuV1 (Martínez et al. 2006). The breeders included in this study had a higher rate of TTSuV1 detection. Although this rate was not different ($p>0.05$) from the detection rates of TTSuVk2 or mixed infections, this result reinforces the hypothesis that breeders may be a source of TTSuV infection for piglets.

Emerging viral infections of pigs have been recently reported in Brazil; these include infections due to rotaviruses B and C (Médici et al. 2011), caliciviruses (Barry, Alfieri & Alfieri 2008, Cunha et al. 2010), kobuvirus (Barry et al. 2011), and hepatitis E virus (Gardinali et al. 2012). The associations of these emerging viruses and of TTSuV with disease in pigs have not been elucidated (Meng 2012). However, the zoonotic potential of these viruses is a major concern, and the surveillance of epidemiological data is important. Moreover, porcine enteric viral infections, emerging or not, require continuous attention in pig herds since intestinal health is directly related to productivity.

## Conclusions

To the best of our knowledge, this is the first study on the age distribution of TTSuV infection to be performed in South America. TTSuV infection was widespread in all stages of pigs maintained within pig production cycle during the period evaluated (2008-2011). Infections due to TTSuV1 and TTSuVk2 were found more frequently in suckling piglets and finisher pigs, respectively, in Brazil. The observation of inversion of TTSuV

genera infection with the age of infected pigs, and the variation in the number of TTSuV genera-induced mixed infections detected might be related to the different biological samples used, and highlights the biological properties of this virus. Studies based on the immune response to TTSuV in hosts may help to elucidate the dynamics of TTSuV genera infection and the pathogenicity of these viruses.

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## 3.3 – **Infecção Simultânea com Cepas Distintas de *Torque teno sus virus* (TTSuV) em Suínos Assintomáticos em Idade de Abate**

## **Simultaneous Infection with Distinct Strains of *Torque teno sus virus* (TTSuV) in Healthy Slaughter-age Pigs**

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SHORT COMMUNICATION

# Simultaneous infection with distinct strains of *Torque teno sus virus* (TTSuV) in healthy slaughter-age pigs

**Raquel de Arruda Leme · Juliane Ribeiro · Alice Fernandes Alfieri · Amauri Alcindo Alfieri**

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**Abstract** The aims of this study were to evaluate *torque teno sus virus* (TTSuV) infection in healthy slaughter-age pigs and to compare the similarities of the untranslated region (UTR) nucleotide (nt) sequences obtained from different biological samples of the same animals. Fifty-eight pigs were evaluated by PCR assay for the presence of TTSuV in paired samples of liver and serum (Group 1, $n$=27) and lung and serum (Group 2, $n$=31). All the pigs were positive for TTSuV infection in the organs sampled and 94.8 % ($n$=55) presented with viraemia. The nt sequence similarities between the Groups 1 and 2 varied from 91.7 % to 96.6 % (TTSuV1) and 91 % to 95 % (TTSuV2). In Group 1, the nt sequence similarities were 93 % (TTSuV1) and 95.4 % (TTSuV2). In Group 2, the nt sequence similarities were 95 % (TTSuV1) and 91 % (TTSuV2). These results revealed the simultaneous infection with distinct strains of TTSuV1 and 2 in healthy pigs at slaughter age.

**Keywords** Liver · Lung · Serum · TTSuV1 and 2 · Multiple infection · PCR

## Introduction

Torque teno virus (TTV) was first isolated in a Japanese patient as the causative agent of an acute post-transfusion hepatitis of unknown aetiology (Nishizawa et al. 1997). TTV has since been identified infecting humans and animals (Kekarainen and Segalés 2012).

Porcine TTV belongs to the family *Anelloviridae*, which is divided into the genus *Iotatorquevirus*, which includes the species *torque teno sus virus 1a* (TTSuV1a) and *torque teno sus virus 1b* (TTSuV1b), and the genus *Kappatorquevirus*, which includes the specie *torque teno sus virus k2* (TTSuVk2) (ICVT 2012).

TTSuV is a non-enveloped virus with a circular, negative-sense, single-stranded DNA (ssDNA) genome (Okamoto et al. 2002). The TTSuV DNA genome is 2.8 kb long and includes four open reading frames (ORFs) and an untranslated region (UTR), which is considered a useful molecular marker in polymerase chain reaction (PCR) assays for TTSuV species differentiation (Segalés et al. 2009; Meng 2012).

TTSuV has not been linked to any specific disease. However, studies have associated important pig pathologies of viral aetiology, like porcine circovirosis due to porcine circovirus-2 (PCV-2), hepatitis E, porcine reproductive and respiratory syndrome, and classical swine fever with TTSuV infection (Kekarainen et al. 2006; Ellis et al. 2008; Savic et al. 2010; Zhu et al. 2012).

TTSuV is known to infect pigs from different geographical regions worldwide, including Asia, Europe, and the Americas (McKeown et al. 2004; Gallei et al. 2010; Savic et al. 2010; Pérez et al. 2011; de Arruda Leme et al. 2012). Marked genetic variability is present among TTSuV species (Cortey et al. 2011), and multiple infections by distinct types and subtypes of TTSuV species in a single pig may occur (Gallei et al. 2010; Huang et al. 2010).

The aims of this study were to evaluate the presences of TTSuV1 and 2 in organs and concomitant viraemia in healthy slaughter-age pigs, and to evaluate the occurrence of simultaneous infection by distinct strains of the same TTSuV species in these same pigs.

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## Materials and methods

Samples were derived from a collection of paired tissue and serum samples collected in two separate pig slaughterhouses under the control of the Inspection Service of Parana state (SIP), Brazil, in July, 2011, for other research purposes and were stored at −80 °C. Pigs were from different herds in this same state; the animals were aged 25 weeks or older and clinically healthy.

A total of 116 samples were included in this study. Paired liver and serum samples of 27 pigs and paired lung and serum samples of 31 pigs from the southern and western regions of Parana state, respectively, were evaluated.

Organs homogenates (10–20 %w/v) were prepared in 0.01 M phosphate-buffered saline (PBS), pH 7.2, and the nucleic acids were extracted using a combination of the phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate nucleic acid extraction methods (Alfieri et al. 2006). Nucleic acid extraction was performed by the silica/guanidinium isothiocyanate nucleic acid extraction method on 200 µl aliquots of the serum samples (Boom et al. 1990).

The presence of TTSuV was determined using a previously described PCR assay for differential detection of species 1 and 2 (Segalés et al. 2009) with modifications (de Arruda Leme et al. 2012). The expected sizes of the amplified products were 305 bp and 252 bp for TTSuV1 and 2, respectively.

Amplified products from 4 different positive pigs were randomly selected for sequencing analysis to confirm the results. Sequence alignment and identity matrix creation were performed using BioEdit software version 7.0.8.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

## Results and discussion

TTSuV was detected in the vast majority of the examined serum, liver, and lung samples. Of all 116 samples analysed, 87.9 % ($n$=102) showed mixed infection by both TTSuV species. Only 9.5 % ($n$=11) of the samples showed single infection (1.7 % for TTSuV1 and 7.8 % for TTSuV2). Three samples tested negative for TTSuV infection (Table 1).

A comparison was made between the sequences found in this study, other sequences derived from pigs in Brazil in other study, and the complete genome sequences (prototypes) available in GenBank. The results are shown in Table 2.

The presence of TTSuV infection in organ samples paired with serum samples of the same healthy adult pigs at the same time has never previously been reported. All 58 pigs were positive for TTSuV infection in the organ samples and 55 (94.8 %) of them presented with viraemia.

Aramouni et al. (2010) suggested that when TTSuV infection occurs before the age of immunocompetence, the host potentially becomes tolerant toward the virus and develops a persistent infection throughout its lifetime. Therefore, the persistence of the TTSuV infection might be related to host immunocompetence. Alternatively, the virus could develop mechanisms to avoid immune responses, such as replicating in low amounts to prevent inflammation of the target tissue (Savic et al. 2010). Another study suggested that after overcoming the infection, the individual suffers re-infection (Sibila et al. 2009). These facts might explain the high number of viral-positive organ samples in this study, as well as the high incidence of viraemia.

Most of the studies that have been performed have reported the prevalence of TTSuV using serum samples (McKeown et al. 2004; Kekarainen et al. 2006; Segalés et al. 2009; Taira et al. 2009; Huang et al. 2010; Cortey et al. 2011; Tshering et al. 2012). The prevalence of TTSuV1 and 2 in serum samples has been demonstrated to increase with the age of the animals (Sibila et al. 2009; Jarosova et al. 2011).

Based on studies that have demonstrated the presence of TTSuV in organs (Bigarré et al. 2005; Aramouni et al. 2010; Gallei et al. 2010; Savic et al. 2010; Pérez et al. 2011; Zhu et al. 2012) it can be concluded that there is no single target tissue for TTSuV. In addition to the increased prevalence of TTSuV viraemia with age, the prevalence of TTSuV infection in organ samples has also been demonstrated to increase with the age of the animals (Aramouni et al. 2010).

None of the previously mentioned studies evaluated serum and organ samples collected at the same time from the same healthy adult animals. A study performed by Tackás et al. (2008) tested liver and intestine samples paired with serum samples for the presence of TTSuV, and the analysed organ samples were found to be infected at lower rates than the serum samples. However, the samples tested were derived from weaned pigs.

Zhu et al. (2012) evaluated TTSuV infection in organ, serum, and stool samples from the same ill pigs at 1.5 to 5 months of age and found the prevalence of TTSuV in tissues much higher than in the stool and serum samples.

**Table 1** TTSuV1 and 2 PCR assay positive results for serum samples paired with their respective tissue samples

| | Paired samples (%) | | | |
|---|---|---|---|---|
| | G1 ($n$=27) | | G2 ($n$=31) | |
| | Serum (%) | Liver (%) | Serum (%) | Lung (%) |
| TTSuV1 | 2 (7.4) | 0 | 0 | 0 |
| TTSuV2 | 0 | 0 | 8 (25.8) | 1 (3.2) |
| TTSuV1+2 | 25 (92.6) | 27 (100) | 20 (64.5) | 30 (96.8) |
| Negative | 0 | 0 | 3 (9.7) | 0 |
| TOTAL | 27 (100) | 27 (100) | 31 (100) | 31 (100) |

**Table 2** Percentage of nucleotide sequence similarities of TTSuV1 and 2 UTR sequences obtained in this study, sequences derived from pigs in Brazil, and the complete genome sequences (prototypes) available in GenBank

| TTSuV strains (Genbank access number) | | Paired samples | | | |
|---|---|---|---|---|---|
| | | G1 | | G2 | |
| | | Serum | Liver | Serum | Lung |
| | | TTSuV1 | | | |
| G1 | Serum (JX083853) | 100 | 93 | 96.3 | 92 |
| | Liver (JX083854) | | 100 | 96.6 | 91.7 |
| G2 | Serum (JX083855) | | | 100 | 95 |
| | Lung (JX083856) | | | | 100 |
| TTV1_BRA11/07 (JQ619841) | | 93.9 | 87.9 | 91.1 | 91.8 |
| Sd-TTV31 (AB076001) | | 91.7 | 91.4 | 94.7 | 96.6 |
| Sd-TTV1p (AY823990) | | 91.4 | 91.1 | 94.3 | 96.3 |
| PTTV1a-VA (GU456383) | | 90.7 | 90.4 | 93.7 | 96 |
| PTTV1b-VA (GU456384) | | 90.4 | 90.1 | 93.4 | 94.7 |
| 20N (1c) (GU570199) | | 90.7 | 90.4 | 93.7 | 95.3 |
| | | TTSuV2 | | | |
| G1 | Serum (JX083857) | 100 | 95.4 | 95 | 91 |
| | Liver (JX083858) | | 100 | 94.6 | 91.9 |
| G2 | Serum (JX083859) | | | 100 | 91 |
| | Lung (JX083860) | | | | 100 |
| TTV2_BRA21/11 (JQ619842) | | 82.6 | 83 | 92.1 | 83.3 |
| Sd-TTV2p (AY823991) | | 93.1 | 95.2 | 94.7 | 90.4 |
| PTTV2b-VA (GU456385) | | 91.1 | 91.6 | 90.3 | 93.6 |
| TTV2_G61(2d) (GU570207) | | 95.5 | 92.8 | 92.7 | 90.8 |
| TTV2_G64(2e) (GU570208) | | 92.3 | 92.4 | 90.7 | 92 |
| TTV2_GE1(2f) (GU570209) | | 90.3 | 90 | 89.5 | 90.4 |
| TTV2_G31(2g) (GU570204) | | 92.3 | 92 | 91.5 | 91.2 |

Only three of the evaluated pigs presented TTSuV-positive serum and organ samples.

In Brazil, Niel et al. (2005) detected TTSuV1 and 2 infections in serum samples for the first time. Ritterbusch et al. (2012) in Santa Catarina state, Brazil, found TTSuV2 infection more frequently than TTSuV1, primarily during co-infection with PCV2, in reproductive organs, semen, ovarian follicular fluid and lymph nodes of adult pigs. Another study showed that early infection with TTSuV1 was significantly higher than TTSuV2 in pig herds from different Brazilian regions by using faecal samples of suckling piglets (de Arruda Leme et al. 2012). No studies on systemic infection by TTSuV have been performed in Brazil until now.

The nt sequence similarities between the strains in this study and other Brazilian strains obtained from faecal samples (de Arruda Leme et al. 2012) varied (87.9 % to 93.9 % for TTSuV1 and 82.6 % to 92.1 % for TTSuV2). Based on the most conserved region of the viral genome, these data reveal the genetic variability among Brazilian TTSuV strains.

The UTR sequence in this study showed variation between the sequences of the same TTSuV species from the same animals (93 % and 95 % for TTSuV1; 95.4 % and 91 % for TTSuV2). The similarities between the same UTR sequence portions of the prototypes were analysed and were also found to vary between each other (93.6 % to 96.8 % for TTSuV1; and 93.5 % to 96.9 % for TTSuV2). Based on the variability of the conserved region nt sequences similarities, these results suggest that the TTSuV strains present in the organ samples in this study were not the same strains that were present in the serum samples.

Multiple infections with both TTSuV species and distinct TTSuV strains within the same species commonly occur (Gallei et al. 2010; Huang et al. 2010). The simultaneous presence of several related but distinct TTSuV strains has been suggested to favour immune evasion by the virus, establishment of infection, and, eventually, disease induction (Gallei et al. 2010).

To our knowledge, this study represents the first description of TTSuV infection in organs paired with serum samples at the same time in the same clinically healthy adult pigs. The results are not restricted to viraemia but also provide data on the distribution of infection in organs. This study showed that

slaughter-age pigs were commonly co-infected with both the TTSuV species. Based on the partial sequences of the conserved region, we concluded that the TTSuV strains in this study differ between the animals and, particularly, that nt sequences obtained from the same pigs differ between each other. This result provides evidence of multiple infections with distinct strains of the same TTSuV species. Further studies are necessary to understand the sanitary risks of the TTSuV infection and the mechanisms of survival and evasion of the host immune responses, tissue tropism, and persistence of the infection by this virus.

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# 4 CONCLUSÕES

- A infecção pelo TTSuV está disseminada em rebanhos suinícolas das principais regiões brasileiras (Sul, Sudeste e Centro-oeste) produtoras de suínos;
- A infecção pelo TTSuV está disseminada em todas as categorias do ciclo de produção de suínos de granjas do estado do Paraná, Brasil;
- A frequência da infecção pelo TTSuV aumenta progressivamente com o aumento da idade dos suínos;
- A infecção pelo TTSuV1 é mais frequente que a infecção pelo TTSuVk2 em leitões de até 3 semanas de idade de rebanhos suinícolas brasileiros;
- A infecção pelo TTSuVk2 é mais frequente em suínos em fase de terminação que a infecção pelo TTSuV1;
- A frequência da infecção pelo TTSuV em fígado e pulmão com concomitante viremia é alta em suínos de granjas do estado do Paraná, Brasil;
- Cepas brasileiras de TTSuV1 e TTSuVk2 apresentam importante variabilidade genética;
- Infecções mistas simultâneas por cepas distintas dos mesmos gêneros de TTSuV são comuns em rebanhos suinícolas do estado do Paraná, Brasil.

# 4 – CONCLUSIONS

- TTSuV infection is widespread in pig herds of the major pig-producing Brazilian (South, Southeast, and Midwest) regions for years;
- TTSuV infection has disseminated in all stages of pig production cycle in farms of Paraná state, Brazil;
- The frequency of TTSuV infection increases progressively with the increasing age of pigs;
- TTSuV1 is more frequent than the TTSuVk2 infection in piglets up to 3 weeks of age in Brazilian pig herds;
- TTSuVk2 is more frequent than the TTSuV1 infection in finisher pigs;
- The frequency of TTSuV infection in liver and lung with concomitant viremia is high in pigs of Paraná state, Brazil;
- TTSuV1 and TTSuVk2 strains from Brazil presents important genetic variability;
- Simultaneous infections with distinct strains of the same TTSuV genera are common in pig herds from Paraná state, Brazil.

# ANEXO

# ANEXO A

## Lista de Reagentes

1. Acetona, P.A. ($CH_3COCH_3$) P.M. 58,08 (Dinâmica®)
2. Ácido acético glacial, P.A. ($CH_3COOH$) P.M. 60,05 (Nuclear®)
3. Ácido bórico ($H_3BO_3$) P.M. 61,83 (Sicalab®)
4. Ácido clorídrico (HCl) P.M. 36,46 (Reagen®)
5. Ácido etilenodiaminotetraácido sal di-sódico – EDTA, P.A. ($C_{10}H_{14}N_2O_8Na_22H_2O$) P.M. 372,24 (Reagen®)
6. Agarose (Invitrogen™ Life Technologies)
7. Água DEPC (Dietil pirocarbonato) (Invitrogen Life Technologies®)
8. Álcool etílico absoluto ($C_2H_2OH$) P.M. 46,07 (Nuclear®)
9. Álcool isoamílico ($(CH_3)_2CHCH_2CH_2OH$) P.M. 88,15 (Synth®)
10. Azul de bromofenol (Sigma®)
11. Bicarbonato de sódio P.A. ($NaHCO_3$) P.M. 84,01 (Biotec®)
12. Borohidreto de sódio P.M. 37,83 (Sigma®)
13. Brometo de etídeo ($C_{21}H_{20}N_3Br$) P.M. 394,3 (Sigma®)
14. Cloreto de cálcio puro ($CaCl_2$) P.M. 110,94 (Invitrogen™ Life Technologies)
15. Cloreto de magnésio 50 mM ($MgC_{l2}$) (Invitrogen Life Technologies®)
16. Cloreto de potássio, P.A. (KCl) P.M. 74,56 (Reagen®)
17. Cloreto de sódio, P.A. (NaCl) P.M. 58,45 (Reagen®)
18. Clorofórmio, P.A. ($CHCl_3$) P.M. 119,38 (Dinâmica®)
19. Dimetil sulfóxido (DMSO) C2H6SO (Sigma®)
20. Dióxido de sílica ($SiO_2$) P.M. 60,08 (Sigma®)
21. Dithiothreitol (DTT-10 mM) (Invitrogen Life Technologies™)
22. DNA Ladder (123 bp) (Invitrogen Life Technologies™)
23. dNTP Set (100 mM), 4 x 250 µL; 25 µmol each (100 mM dATP Solution, 100 mM dCTP Solution, 100 mM dGTP Solution, 100 mM dTTP Solution) (Invitrogen Life Technologies™)
24. Dodecil sulfato de sódio – Lauril Sulfato de Sódio – SDS ($C_{12}H_{25}NaO_4S$) P.M. 288,38 (Synth®)
25. Fenol ($C_6H_5OH$) P.M. 94,11 (Invitrogen Life Technologies™)
26. Fosfato de sódio dihidratado ($Na_2HPO_4$ . $2H_2O$) P.M 177,99 (Merck®)

27. Fosfato de sódio monobásico ($NaH_2PO_4 . 2H_2O$) P.M. 155,99 (Reagen®)
28. Fosfato de sódio dibásico anidro ($Na_2HPO_4$) P.M. 141,96 (Synth®)
29. Glicose ($C_6H_{12}O_6$) P.M. 180,16 (Reagen®)
30. Hidróxido de sódio, P.A. (NaOH) P.M. 40,00 (Dinâmica®)
31. Hidroximetil amino metano – TRIS 99% P.M. 121,14 (Inlab®)
32. Isotiocianato de guanidina P.M. 118,16 (Gibco BRL®)
33. Metanol P.A. ($CH_3OH$) P.M. 32,04 (Allkimia®)
34. Oligonucleotídeo iniciador (*primer*) forward-TTSuV1 (forward; 5′-CGGGTTCAGGAGGCTCAAT -3′; nucleotide (nt) 9-27) Segalés *et al*. (2009) - 200 pmol (Invitrogen Life Technologies®)
35. Oligonucleotídeo iniciador (*primer*) reverse-TTSuV1 (reverse; 5′-GCCATTCGGAACTGCACTTACT -3′; nucleotide (nt) 313-292) Segalés *et al*. (2009) - 200 pmol (Invitrogen Life Technologies®)
36. Oligonucleotídeo iniciador (*primer*) forward-TTSuV2 (forward; 5′-TCATGACAGGGTTCACCGGA -3′; nucleotide (nt) 1-20) Segalés *et al*. (2009) - 200 pmol (Invitrogen Life Technologies®)
37. Oligonucleotídeo iniciador (*primer*) reverse-TTSuV2 (reverse; 5′-CGTCTGCGCACTTACTTATATACTCTA -3′; nucleotide (nt) 252-226) Segalés *et al*. (2009) - 200 pmol (Invitrogen Life Technologies®)
38. PCR-buffer (10x) (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen Life Technologies™)
39. *Platinum* Taq DNA Polymerase recombinant 500 units (Invitrogen Life Technologies™)
40. Proteinase K (10mM Tris; 1mM ethylenediamine tetra-acetic acid [EDTA]; 0.5% Nonidet P40; 1% sodium dodecyl sulfate [SDS]; 0.2mg/ml proteinase K) (Invitrogen Life Technologies™)
41. QuantIT ™ dsDNA BR assay kit (Invitrogen Life Technologies™)
42. Sacarose, P.A. – sucrose ($C_{12}H_{22}O_{11}$) P.M. 342,31 (Reagen®)
43. Triton x-100
44. Vermelho de fenol ($C_{19}H_{14}O_5S$) P.M. 354,38 (Reagen®)

# ANEXO B

## Soluções e Tampões

- **Diluição dos primers**

PCR para detecção da região UTR do genoma do TTSuV1

- *Primer foward-TTSuV1*
Sequência: 5'- CGGGTTCAGGAGGCTCAAT - 3'
Posição: 9-27
Concentração: 22,81 nmoles
Data de fabricação: Jun / 2011

22,81 x 1000 = 22810 pmoles
228100 / 200 = 114,05

*Primer* mãe (200 pmol/μL): ressuspender em 114,05 μL de água MilliQ autoclavada para obtenção de solução 10x [ ]

*Primer* uso 1x [ ] (20 pmol/μL): diluir o *primer* mãe (1:10) em água MilliQ autoclavada

- *Primer reverse-TTSuV1*
Sequência: 5'- GCCATTCGGAACTGCACTTACT – 3'
Posição: 313-292
Concentração: 21,11 nmoles
Data de fabricação: Jun / 2011

21,11 x 1000 = 21110 pmoles
21110 / 200 = 105,55

*Primer* mãe (200 pmol/μL): ressuspender em 105,55μL de água MilliQ autoclavada para obtenção de solução 10x [ ]

*Primer* uso 1x [ ] (20 pmol/μL): diluir o *primer* mãe (1:10) em água MilliQ autoclavada

PCR para detecção da região UTR do genoma do TTSuV2

- *Primer foward-TTSuV2*
Sequência: 5' – TCATGACAGGGTTCACCGGA – 3'
Posição: 1-20
Concentração: 17,41 nmoles
Data de fabricação: Jun / 2011

17,41 x 1000 = 17410 pmoles
17410 / 200 = 87,05

*Primer* mãe (200 pmol/μL): ressuspender em 87,05μL de água MilliQ autoclavada para obtenção de solução 10x [ ]

*Primer* uso 1x [ ] (20 pmol/μL): diluir o *primer* mãe (1:10) em água MilliQ autoclavada

- *Primer reverse-TTSuV2*
Sequência: 5' – CGTCTGCGCACTTACTTATATACTCTA – 3'
Posição: 252-226
Concentração: 21,03 nmoles
Data de fabricação: Jun / 2011

21,03 x 1000 = 21030 pmoles
21030 / 200 = 105,15

*Primer* mãe (200 pmol/μL): ressuspender em 105,15 μL de água MilliQ autoclavada para obtenção de solução 10x [ ]

*Primer* uso 1x [ ] (20 pmol/μL): diluir o *primer* mãe (1:10) em água MilliQ autoclavada

- **Diluição de dNTP**

  - solução estoque (100 mM) – 100 μL de cada dNTP
  - solução uso (10 mM) – 10 μL da solução estoque + 90 μL de água MilliQ autoclavada

- **Fenol / clorofórmio – álcool isoamílico (25:24:1)**

  - 25 mL fenol saturado em água
  - 24 mL clorofórmio
  - 1 mL álcool isoamílico

- **Gel de agarose 1%**

  - 0,5 g agarose
  - 50 mL de tampão TBE 1x
  - 20 μL de brometo de etídio

- **Hidratação da sílica**

  - 60 g de sílica (SIGMA®)
  - Adicionar 500 mL de água MilliQ autoclavada
  - Agitar lentamente e manter em repouso durante 24 h
  - Por sucção, desprezar 430 mL do sobrenadante
  - Ressuspender a sílica em 500 mL de água bidestilada
  - Manter em repouso durante 5 h para sedimentar
  - Desprezar 440 mL do sobrenadante
  - Ajustar o pH (pH 2,0)
  - Aliquotar e autoclavar

- **SDS 10%**

  - 5 g dodecil sulfato de sódio – Lauril sulfato de sódio – SDS ($C_{12}H_{25}NaO_4S$)
  - água bidestilada q.s.p. 50 mL

- **Solução L6**

  - 120 g de isotiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4
  - 22 mL de EDTA 0,2 M pH 8,0
  - 2,6 g de Triton 100

- **Solução L2**

  - 120 g de isotiocianato de guanidina (GUSCN)
  - 100 mL de TRIS-HCl 0,1 M pH 6,4

- **Tampão de amostra para eletroforese em gel de agarose**

  - azul de bromofenol 0,25%
  - sacarose – sucrose ($C_{12}H_{22}O_{11}$) 45%
  - água bidestilada q.s.p.100 mL

- **Tampão de corrida – TBE (Tris – Ácido bórico – EDTA) 10x**

  - Tris 0,89 M
  - Ácido bórico 0,89 M
  - EDTA 0,02 M
  - água bidestilada q.s.p. 1 litro
  - ajustar o pH (pH 8,4)

- **Tampão Fosfato Salina – PBS**

  - 137 mM Cloreto de sódio (NaCl)
  - 3 mM Cloreto de potásso (KCl)
  - 8 mM Fosfato de sódio dibásico anidro ($Na_2HPO_4$)
  - 15 mM Potássio fosfato monobásico ($KH_2PO_4$)
  - Água ultrapura autoclavada q.s.p. 500 mL

# ANEXO C

## Protocolos de Técnicas

- **Extração do ácido nucléico pela associação das técnicas fenol/clorofórmio/álcool isoamílico e sílica/isotiocianato de guanidina**

1. *Suspensão fecal – extração bruta*

- 100 µL ou 100 mg de fezes
- 500 µL de PBS
- Vortexar
- Centrifugar a 5000 x *g* / 3 min
- Utilizar 400 µL do sobrenadante para extração

2. *Suspensão de tecido – extração bruta*

- 1,5 g do fragmento de órgão (fígado ou pulmão)
- Macerar
- 15 mL PBS
- Vortexar
- Centrifugar a 3000 x g / 10 min
- Utilizar 250 µL do sobrenadante para extração

- 250 µL da suspensão do fragmento de órgão
- 10 µL de tampão de lise (Proteinase K)
- Homogeneizar em *vortex*
- Incubar a 56°C / 30 min
- Utilizar todo o volume para a extração do ácido nucléico

*3. Extração do ácido nucléico*

Fase I – Fenol

- 400 µL da suspensão fecal OU 250 µL da suspensão de tecido
- Adicionar 40 µL de SDS 10% OU 25 µL de SDS 10 %
- Homogeneizar em *vortex*
- Banho-maria 56 °C /20 min
- Centrifugar 10.000 x *g* /30 s
- Adicionar 400 µL de fenol/clorofórmio-álcool isoamílico (25:24:1)
- Homogeneizar em *vortex*
- Banho-maria 56 °C /15 min
- Homogeneizar manualmente por 15 s
- Centrifugar 10.000 x *g* /10 min
- Recolher o sobrenadante em outro microtubo

Fase II – Sílica / isotiocianato de guanidina

- Adicionar 500 µL da solução L6
- Adicionar 25 µL de sílica hidratada
- Homogeneizar em *vortex*
- Agitar em temperatura ambiente /30 min
- Centrifugar 10.000 x *g* /30 s
- Desprezar o sobrenadante em solução contendo NaOH 10 M
- Adicionar 500 µL de solução L2
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s
- Desprezar o sobrenadante em solução contendo NaOH 10 M
- Adicionar 500 µL de solução L2
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s
- Desprezar o sobrenadante em solução contendo NaOH 10 M
- Adicionar 1000 µL de etanol 70% gelado
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s

- Desprezar sobrenadante em descarte comum
- Adicionar 1000 µL de etanol 70% gelado
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s
- Desprezar sobrenadante em descarte comum
- Adicionar 1000 µL de acetona P.A. gelada
- Homogeneizar em *vortex*
- Centrifugar 10.000 x *g* /30 s
- Desprezar sobrenadante
- Secar o *pellet* em termo bloco a 60ºC (aproximadamente 2 min) ou banho-maria a 56ºC (15 min)
- Adicionar 50 µL de água DEPC
- Homogeneizar em *vortex*
- Banho-maria 56ºC/15 min
- Homogeneizar em *vortex*
- Centrifugar 13.000 x *g* /4 min
- Recolher o sobrenadante em microtubo de 500 µL
- Estocar -20ºC até a utilização

- **Reação em cadeia pela polimerase (PCR)**

- Mix de PCR para detecção parcial da região UTR genoma do TTSuV1

| **Reagentes** | **Volume (µL)** |
|---|---|
| *Buffer* 10 x (pH 8,4) | 1,5 |
| $MgCl_2$ | 0,8 |
| dNTP (2,5 mM) | 2,5 |
| *Platinun*®Taq DNA Polymerase (5U/µL) | 0,15 |
| Primer F-TTSuV1 (20 pmol) | 0,5 |
| Primer R-TTSuV1 (20 pmol) | 0,5 |
| Água | 10,05 |
| Volume final | 20 |

- Mix de PCR para detecção parcial da região UTR genoma do TTSuV2

| **Reagentes** | **Volume (μL)** |
|---|---|
| *Buffer* 10 x (pH 8,4) | 1,5 |
| $MgCl_2$ | 0,8 |
| dNTP (2,5 mM) | 2,5 |
| *Platinun*®Taq DNA Polymerase (5U/μL) | 0,15 |
| Primer F-TTSuV2 (20 pmol) | 0,5 |
| Primer R-TTSuV2 (20 pmol) | 0,5 |
| Água | 10,05 |
| DNA | 4 |
| Volume final | 20 |

- Ciclos de tempo e temperatura da PCR

| **Reação** | **Temperatura (°C)** | **Tempo (min)** | **Nº de Ciclos** |
|---|---|---|---|
| Desnaturação | 94 | 5 | 1 |
| Desnaturação | 94 | 1 | 40 |
| Anelamento | 54 | 1 | 40 |
| Extensão | 72 | 1 | 40 |
| Extensão final | 72 | 5 | 1 |

- **Eletroforese em gel de agarose a 1%**

- 0,5 g de agarose
- 50 mL TEB *buffer* (Tris 89mM; ácido bórico 89 mM; EDTA 2mM) pH 8,4
- 20 μL de brometo de etídeo (0,5 μg/mL)

São utilizados 5 μL do amplicon e 1 μL do tampão de amostra. A eletroforese sob voltagem (100V) e amperagem (80A) constantes ocorre em aproximadamente 50 min.

- **Purificação de produto de PCR excisado do gel**

1. Pesar o fragmento excisado do gel em microtubo de 1,5 mL.
2. Adicionar 10 μL do *Capture buffer type* 2 para cada 10 mg de gel.
3. Incubar o tubo a 60°C / 15 min, homogeneizando a cada 3 min.

4. Centrifugar a 13.000 x *g* / 30s
5. Transferir 600 μL da amostra com o *Capture buffer type* 2 para um tubo coletor com coluna
6. Incubar em temperatura ambiente por 1 min
7. Centrifugar a 13.000 x *g* / 30s
8. Descartar o filtrado e recolocar a coluna no mesmo tubo.
9. Adicionar 500 μL do *Wash buffer type 1* na coluna com tubo coletor
10. Centrifugar a 13.000 x *g* / 30s
11. Descartar o filtrado e transferir a coluna para um microtubo de 1,5 mL.
12. Adicionar 30 μL do *Elution buffer type 6*
13. Incubar a temperatura ambiente por 1 min.
14. Centrifugar a 13.000 x *g* / 1 min.
15. Estocar o fragmento de DNA purificado a -20°C.

- **Quantificação de produto de PCR**

(Certificar-se de que todos os reagentes estão em temperatura ambiente)

1. Preparar a solução Quant-iT™ *Working Solution* diluindo o reagente Quant-iT™ em *Buffer* Quant-iT™ 1:200. São necessários 200 μL desta solução por amostra e para os padrões 0 e 100.

2. Homogeneizar em *vortex*.

3. No microtubo das amostras adicionar 198 μL da solução Quant-iT™ *Working Solution* a 2 μL do fragmento de DNA purificado.

4. No microtubo do padrão 0 adicionar 190 μL da solução Quant-iT™ *Working Solution* a 10 μL do padrão 0.

5. No microtubo do padrão 100 adicionar 190 μL da solução Quant-iT™ *Working Solution* a 10 μL do padrão 100.

6. Homogeneizar os microtubos em *vortex* por 2-3 s

7. Incubar os microtubos em temperatura ambiente por 2 min

8. Realizar a leitura usando Qubit™ fluorometer (Invitrogen™ Life Technologies, EUA)

9. Multiplicar pelo fator de diluição para determinar a concentração correta da amostra

# ANEXO D

## Lista de *Softwares*

- Electropherogram quality analysis - Phred e CAP3

(http://asparagin.cenargen.embrapa.br/phph/)

- BLAST The Basic Local Alignment Search Tool

(http://blast.ncbi.nlm.nih.gov/Blast.cgi)

- MEGA package software version 4.1

(http://www.megasoftware.net/mega4/mega41.html)

- BioEdit software version 7.0.9.0

(http://www.mbio.ncsu.edu/bioedit/bioedit.html)

**“Aprendamos a entesourar os dons da vida, respeitando os ensinamentos que o mundo nos impõe, na certeza de que, entre a humildade e o trabalho, alcançaremos, um dia, os cimos da glória eterna.”**

**Por minha avó, Odette**

**(autor deconhecido).**